UNIVERSIDAD AUTÓNOMA DE MADRID

Faculty of Science

Department of Molecular Biology

Characterization of pathogenicity factors of a lethal influenza A(H1N1)09 pandemic viral isolate

Doctoral Thesis

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Madrid 2016

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This work have been done at the Nacional Center of Biotechnology (CNB- CSIC) at the department of Cellular and Molecular Biology thanks to La Caixa International PhD Fellowship, Plan Nacional de Investigacion Científica, Desarrollo e Innovacion Tecnologica (BFU2011-26175 and BFU2014-57797-R) and the Ciber de Enfermedades Respiratorias (CIBERES).

Za moju porodicu

For Carmen and Suresh

Acknowledgements

First, I would like to thank my thesis directors Amelia and Ana, who have guided me on this work for the last 3 years, helping me make it the way it is today. I thank them for the opportunity to let me grow and learn, think outside the box, be creative and teaching me not be afraid to question.

My thanks to my lab mates (Noelia, Laura, Paloma and Ale) as well, for always being there for me, going out of their way in helping me, and for just giving love and support.

Gema, Victoria, Lidia, Gines and Diego, thank you for making every unbearable day (and you know there have been many of them) more bearable and for finding a way to remind me that sometimes what is important above everything else is what I believe, think and know.

Thanks to Lili, Noelia, Silvia and Ernie who helped me more than they could know just by listening.

Thanks to my crew, my peeps, 241 lab both old and new members. Bea, Massi, Lidi, Evita, Yago, Alex, Victor, Alvarus and Luis Angel, who adopted me from the day one, making me feel at home. I can honestly say that you are some of the best people I have ever met. I am so grateful for your friendship, your support and genuine care. Thank you for the unforgettable moments, talks and some of my best times spent in CNB - our lunch time together.

Thanks to my ex 211 lab. Elvita, Noe, Coloms, Clau, Ynes, Jero and Alex. My first contact with Spanish people, culture and my first contact with science. Thank you for making me feel welcome and accepting me from the moment I walked in. Thank you for making science fun and amazing, for making me forget about time while working and be excited to wake up every morning and go to work. Thank you for the endless talks about everything from Carl Sagans' blue dot, hyper mutant bacterial strains, Rafaels' english skills in "Aquarius" to Star Wars films. I loved every moment I was part of something great, 211. Mutadores 4ever.

Thanks to my little Spanish-Indian-Cuban family. You know I would not be able to make it without you. Carmendy, Su, Clau and Raj, you are the best part of the last 4 years and at the end, you make it all worth it. Thank you for being there every step of the way, pushing me to overcome all the obstacles and to never give up.

Carmendy and Su, this thesis is as much as yours as it is mine. If I would have to do everything all over again (and you know, you know what that implies) I would do it in a blink of an eye if it would mean having you as my friends. My heart and soul goes out to you. I cannot thank you enough, and no words can describe how much I appreciate all that you have done. I am proud to call you my friends, although you are much more than that, you are my family tied by kindness, care and love. I wish there were more people like you in the world - what a place it would be! You make it all better, you make me better, I love you.

Za moju porodicu. Kada sam polazila u Madrid, znam da sam jedva čekala da ove 4 godine prodju. U međuvremenu sam upoznala neke divne, predivne ljude, pune vrlina, znanja i razumevanja (nekada nisam bila te sreće), stekla prijatelje za ceo život i videla i onu drugu, ne tako svetlu stranu ljudi. Zbog svog iskustva koje sam stekla u proteklih 4 godine želim da vam kažem:

Nati, mama, tata, liko, dekane i Yahir,

Hvala vam sto ste me naučili da budem čovek, dobar čovek, da radim ispravno, govorim šta mislim, da se ne kitim tuđim perjem, ne mešam u stvari koje me se ne tiču, ignorišem glupost i poštujem razum. Hvala vam što ste me naučili da ne budem nemi posmatrač nepravde, da ne gazim preko drugih da bih zadovoljila svoje sitne potrebe, da nisu svi ljudi isti, da biram svoje prijatelje, verujem u sebe, ma šta neki ljudi pričali i da znam da su neznaje i zavist pošast.

Hvala vam što znate sve ovo i što ste mene i seku naucili da budemo kao vi. Hvala vam što ste uvek bili tu, svaki dan, svaki sat, svaki tren. Hvala vam za najlepši život koji ste nam pružili. Ovo nije samo moj uspeh, ovo je vaš uspeh. Volim vas sve mnogo, mnogo, mnogo.

Nati, mama, tata, liko, dekane i Yahir, ovo je za vas.

Table of Content

Abbreviations

aa amino-acid

A549 human type II alveolar epithelial cells

AH1N1pdm09 pandemic 2009 A H1N1 virus

AM Alveolar Macrophages

Amp Ampicillin

APC Antigen Presenting Cells

APDS Acute Pulmonary Distress Syndrome

AV node Atrioventricular node

CAL A/California/04/09 virus

CAL-HA HA S127L A/California/04/09 virus

CAL-M M1 86S-M2 30N A/California/04/09 virus

CAL-M/PA M1 86S-M2 30N-PA D529N A/California/04/09 virus

CAL-PA PA D529N A/California/04/09 virus

CAL-PB2 PB2 A221T A/California/04/09 virus

CAL-PB2/PA PB2 A221T-PA D529N A/California/04/09 virus

CARD Caspase Activation and Recruitment Domains

Cardif CARD-containing adaptor protein

CAT Chloramphenicol Acetyltransferase

Ccl19 Chemokine (C-C motif) ligand 19

Ccl19 Chemokine (C-C motif) ligand 19

Ccr5 C-C chemokine receptor type 5(CD195)

CHD1, 6 Chromodomain Helicase DNA-binding protein 1, 6

CLPs C-type Lectin Receptors

CMP-1 Calmodulin-binding protein

CNIC Centro Nacional de Investigaciones Cardiovasculares

cRNA Complementary RNA

Csf3 Colony-stimulating factor 3

CVB Coxsackievirus B

Cxcl10 Chemokine (C-X-C motif) ligand 10 (IP10)

Cxcl11 Chemokine (C-X-C motif) ligand 11

Cxcl13 chemokine (C-X-C motif) ligand 13

Cxcl9 Chemokine (C-X-C motif) ligand 9

DC Dendritic cell

DGs Defective Genomes

DIs Defective Interfering RNAs

DMEM Dulbecco's modified Eagle's medium

DNase DNA ribonuclease

Dpi Day post-infection

dsRNA Double -stranded RNA

ECG Electrocardiogram

EDTA Ethylenediaminetetraacetic acid

eIF2α Eukaryotic initiation factor 2α-subunit

eIF4G Eukaryotic translation initiation factor 4G

F strain virus isolated from patient with fatal outcome

FBS Fetal bovine serum

HA Hemagglutinin

hCLE/C14orf166 chromosome 14 open reading frame 166

HEK293T 293T human embriotic kidney cells

HIV Human Immunodeficiency Virus

HPAIV Highly Pathogenic Avian Influenza Viruses

IAV Influenza A Virus

IFITMs IFN induced transmembrane proteins

IFN α Interferon α

IFNAR IFN-α/β receptor

IFN-β Interferon β

IFN-λ1 Interferon λ1

IL1B Interleukin 1 beta

IL-6 Interleukin 6

IL-8 Interleukin 8

ILs Interleukins

IP10 Interferon-gamma-inducible protein 10 (Cxcl10)

IRF3,7,9 Interferon Regulatory Factor 3,7,9

ISG56 Interferon Stimulated Gene 56 protein

ISGF3 Interferon Stimulated Gene Factor 3

ISGs Interferon Stimulated Genes

ISRE Interferon-Sensitive Responsive Element

IκB nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha

Jak1 Janus kinase

LB Luria-Bertani medium

LD_{50} Viral dose that causes 50% of mortality

LGP2/RLR3 RIG-I-like receptor 3

LTA Lymphotoxin-alpha(TNF-β)

Ly6G Lymphocyte antigen 6 complex locus G6D

M strain virus isolated from patient with mild symptoms

M1 Matrix Protein 1

M2 Matrix Protein 2

MAVS Mitochondrial Antiviral Signaling Proteins

MCP-1 Monocyte Chemoattractant Protein-1

MDA5 Melanoma differentiation associated factor 5

MDCK Madin-Darby canine kidney cells

mDCs myeloid dendritic cells

mg milligram

ml milliliter

MIP-1α Macrophage Inflammatory Proteins 1α

MIP-1β Macrophage Inflammatory Proteins 1β

MOI Multiplicity of infection

MORC3- MORC family CW-type zinc finger 3

mRNA Messenger RNA

MxA Myxovirus resistance gene A protein

MyD88 Myeloid differentiation primary response gene 88

NA Neuraminidase

NEP Nuclear Export protein

NF-κβ Nuclear Factor kappa-light-chain-enhancer of activated B cells

NK Natural killer cells

NKT Natural killer T cells

NLRP3 NOD-LRR-and pyrin domain-containing 3

nm nanometer

NOD-LRR Nucleotide-binding Oligomerization Domain Leucine-Rich Repeat

NP Nuclear Protein

NS1 Non-structural 1 protein

OAS 2'-5' olygoadenylate synthetase

PA Polymerase acidic protein

PABP1 PolyA binding protein 1

PAMPs Pathogen Associated Molecular Patterns

PB1 Polymerase basic 1 protein

PB2 Polymerase basic 2 protein

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PFU Particle forming units

PKR Protein kinase R

PRRs Pathogen Recognition Receptors

RANTES/Ccl5 Chemokine (C-C motif) ligand 5

RIG-I Retinoic-acid Inducible Gene I-like receptors

RIP1 Receptor-Interacting serine/threonine-Protein kinase 1

RLR RIG-I like receptors

RNA Ribonucleic Acid

RPM Reads per million

rRNA ribosomal RNA

RSV Respiratory Syncytial Virus

SA node Sinoatrial node

SA Sialic acid

SDS Sodium dodecyl sulphate

ssRNA Single Stranded RNA

STAT1,2 Signal Transducer and Activator of Transcription

TBK1 TANK binding kinase 1

TIR Toll-IL-1 receptor

TLR 2,3,4,7,8,9 Toll-like receptors 2,3,4,7,8,9

TNF tumor necrosis factor

TNF-α Tumor necrosis factor α

TRAF6 TNF receptor associated factor

TRIF TIR-domain-containing adapter-inducing interferon-β

TRIM25 Tripartite Motif containing 25

Tyk2 Tyrosine kinase

VISA Virus-Induced Signalling Adapter

vRdR Viral RNA dependent RNA polymerase

vRNA Viral RNA

vRNPs Viral Ribonucleoproteins

wt wild type

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Summary

Influenza virus is responsible for seasonal outbreaks that result in more than 5 million hospitalization and 500 000 deaths every year. Analysis of two clinical AH1N1pdm09 pandemic isolates originated from a patient with mild (M strain) illness and a patient that deceased (F strain) from influenza related complication during the 2009 pandemic, highlighted three amino-acid changes PB2 A221T, PA D529N and HA S127L in the F isolate, that might be responsible for the increased virulence of this strain *in vitro* and *in vivo*. Furthermore, F isolate was able to accumulate less defective genomes (DGs) than M virus during the infection in cell culture as well as in animal model. Mutations found in F strain and their effects in F virus pathogenicity were studied by introducing its changes in A/California/04/09 (CAL) virus backbone. Although these mutations did not affect viral growth or polymerase activity *in vitro*, we found that PB2 A221T and HA S127L attenuated while PA D529N increased the already described pathogenicity of CAL virus *in vivo*. Besides 100 fold decreased LD_{50} , CAL-PA D529N virus showed high replication rates in lungs of infected animals and strong innate immune response cell influx (neutrophils, dendritic cells, monocytes, alveolar macrophages) and inflammation in early infection (2 day post-infection). Furthermore, CAL-PA D529N virus showed great potential of viral spread to extra-pulmonary organs. Animals infected with CAL-PA D529N virus had infectious viral particles in heart tissue for a prolonged period of time (up to 4 days of infection) and at higher frequency (80% of animals) than CAL infected animals. We demonstrated that beside high viral titre, viral mRNAs of NEP, only present in cells with ongoing infection, were present in heart tissue of animals infected with CAL- PA D529N virus. Analysis of electrocardiogram (ECG) monitorization of infected animals showed that those infected with CAL-PA D529N virus suffered from systolic bradycardia and conduct cardiac defects. Accordingly with the data we obtained from studying F strain, CAL-PA D529N virus have low accumulation of defective genomes in cell culture as well as low induction of antiviral genes (MxA, ISG56). Additionally, our work shows that PA D529N mutation is able to reduce the accumulation of DGs in viruses with high DG accumulation capacity.

In summary, PA D529N is responsible for increased pathogenicity of F strain. As a consequence of this mutation virus accumulates less DGs which partially delays timely antiviral response. This would allow virus to remain undetected by the infected cells for a relatively short but sufficient time period to enable viral replication. High replication rates in lungs of CAL-PA D529N virus and efficient viral spread into extra-pulmonary organs in animals increase its pathogenicity potential. These findings give us an insight into complex pathogenesis of influenza A viruses infections and the different ways it can affect final outcome of disease.

Resumen

El virus de la gripe es responsable de brotes estacionales que suponen más de 5 millones de hospitalizaciones y 500 000 muertos cada año. El análisis de dos aislados clínicos de la pandemia del 2009 (AH1N1pdm09), uno aislado de un paciente con síntomas leves (M) y otro de un paciente fallecido (F), puso de manifiesto la presencia de tres cambios de amino acido en el asilado F (PB2 A221T, PA D529N, HA S127L), que podrían ser los responsables de la virulencia exacerbada de este aislado. Además el aislado F presentó menor acumulación de genomas defectivos (DGs), que el aislado M tanto en viriones aislados de células en cultivo como de pulmones de ratones infectados. El papel de las mutaciones encontradas en el aislado F como posibles responsables de su patogenicidad, fue estudiado mediante la introducción de estos cambios en virus recombinantes de la cepa pandémica de referencia A/California/04/09 (CAL). Ninguna de las mutaciones afectaron la cinética de crecimiento o la actividad de la polimerasa viral *in vitro,* pero PB2 A221T y HA S127L atenuaron y PA D529N incrementó la patogenicidad del virus CAL en el modelo de ratón. El virus recombinante CAL-PA D529N redujo 100 veces la LD50 de los ratones infectados, mostró alta tasa de replicación en los pulmones, potente reclutamiento de células de respuesta inmune innata (neutrófilos, células dendríticas, monocitos, macrófagos alveolares) e inflamación a tiempo temprano de infección (2 días post-infección). Además, el virus CAL-PA D529N mostró un gran potencial de infección en tejidos extrapulmonares, así los ratones infectados con este virus presentaron partículas infectivas en los corazones durante más tiempo (hasta 4 días post-infección) y a mayor frecuencia (80% de los animales infectados) que los ratones infectados con el virus CAL. La presencia de partículas infectivas en los corazones, fue el resultado de replicación viral en este órgano, ya que se detectó en ellos la presencia del mRNA de la proteína NEP, que únicamente es detectada como consecuencia de replicación. La monitorización del electrocardiograma de los ratones infectados con el virus CAL-PA D529N mostró la presencia de bradicardia sistólica y defectos en la conductancia. Similarmente al aislado F, el virus CAL-PA D529N acumuló una baja cantidad de DGs en los viriones aislados de células infectadas y de acuerdo con ello una baja inducción de genes antivirales (MxA, ISG56). Adicionalmente, observamos que la mutación PA D529N reduce la producción de DGs en virus con alta tasa de acumulación de estos genomas defectivos.

En conclusión el cambio PA D529N es el responsable de la patogenicidad del aislado F. El virus recombinante conteniendo este cambio, acumula una baja cantidad de DGs, lo que retrasa la respuesta antiviral. Este hecho permitiría al virus permanecer indetectable por la célula infectada por un tiempo relativamente corto pero suficiente para permitir la replicación viral. Altas tasas de replicación en pulmón en los ratones infectados con CAL-PA D529N y una eficiente dispersión a tejidos extrapulmonares incrementarían su potencial de patogenicidad. Estos resultados nos aportan datos sobre el complejo mecanismo de patogenicidad del virus de la gripe y las diferentes vías que intervienen en el resultado final de la infección.

1. Influenza virus and flu

Acute respiratory infections continue to be the main cause of acute illnesses worldwide and influenza A virus is one of the major contributors. Influenza virus is responsible for seasonal outbreaks that result in more than 5 million hospitalization and 500 000 deaths every year. Just during the last century it caused several pandemic outbreaks with severe public health and economic impact *(WHO)*.

Influenza virus belongs to the *Orthomixoviridae* family that consists out of 6 genera influenza A, B, C, (Infectious salmon anemia), Thogoto virus and newly described D influenza viruses *(Hause, Collin et al. 2014)*. Influenza A and B viruses co-circulate in human population and are responsible for the influenza, an acute respiratory disease infection of both upper and lower respiratory tract causing seasonal epidemics. Influenza type C infections cause mild respiratory illness and are not thought to cause epidemics. Usual symptoms of influenza infection are tracheitis and marked myalgia as well as fever which last up to 5 days *(Couch 1996)*.

Besides pulmonary complication, patients with severe flu infection show various cardiac dysfunctions. Myocarditis and pericarditis are well-recognized cardiac problems that may lead to heart impairment and cause high mortality by influenza virus infection *(Mamas, Fraser et al. 2008)*. It has been reported the infection of both left and right ventricles in heart muscle of one patient who underwent cardiac arrest in very early stage of infection (3rd day of infection) indicating that ability of viral strain to infect and induce inflammation in heart tissue greatly increase its pathogenicity *(Komai, Nakazawa et al. 2011)*. Recent studies suggest that almost 10% of patients suffer from some cardiac abnormalities during the infection *(Jeyanathan, Overgaard et al. 2013)*. All this implies that, influenza A virus H1N1 may induce cardiac abnormalities which may have serious consequences on patients recovery.

Renal complications followed by influenza A virus (IAV) infection are very rare and found in critically ill patients. These dysfunctions include acute kidney injury, rhabdomyolysis, hemolytic uremic syndrome etc. that can be followed by secondary bacterial kidney infection and affect patients overall medical condition. Disseminated intravascular coagulation and acute tubular necrosis were observed in fatal pandemic 2009 H1N1 (AH1N1pdm09) cases *(Watanabe 2013)*.

Infection with A(H1N1)pdm09 strains caused rare but very serious neurological complications, especially in children. Complications include febrile seizures (epilepsy, nonketotic hyperglycinemia, anoxic encephalopathy), encephalitis etc. *(Frobert, Sarret et al. 2011)*.

In summary, viral spread of influenza H1N1 virus plays a key role in pathogenicity of particular virus. Ability of clinical strains to spread and infect non-target tissues causes severe and serious medical conditions and health disorders. Critically ill patients showed broad spectrum of medical complications that greatly impact their clinical picture and recovery.

Seasonal epidemics are caused by both influenza A and B subtypes which are distinguished by two viral glycoproteins (hemagglutinin-HA, neuraminidase-NA). This is due to highly variable superficial viral glycoproteins (HA, NA) that allow them to escape the recognition by host virus specific antibodies. Gradual accumulation of changes in these proteins that retain a degree of serologic relationship with the currently prevailing strain, are called antigenic drift *(Figure 1a)*.

Introduction of novel subtype with antigenically distinct superficial molecules showing no serologic relationship with strains circulating at the time is known as antigenic shift *(Figure 1b)*. These late changes have been observed only in type A virus since it remains inside its reservoirs in various animals besides humans (pigs, horses, birds and other mammals). Efficient transmission from human to human of these antigenically distinct strains cause pandemic outbreaks since neutralizing antibodies to new reassortant virus are absent in the human population.

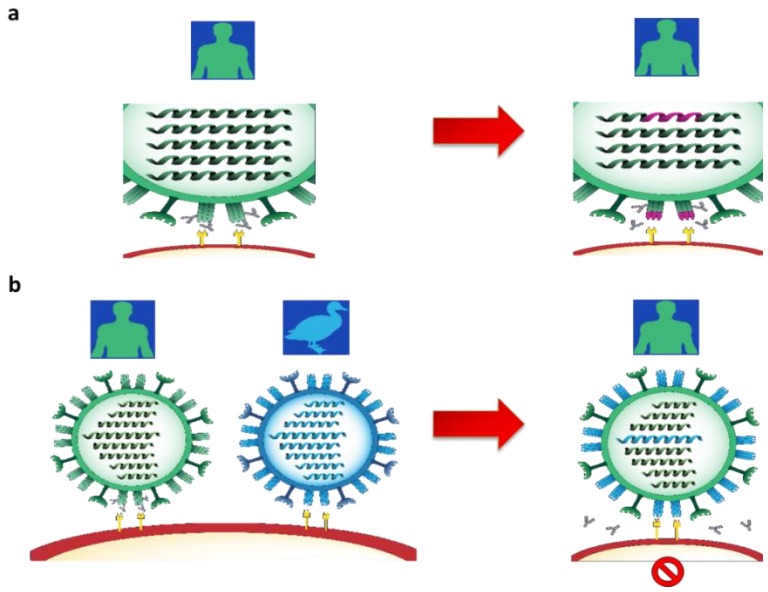


Figure 1. Seasonal and pandemic viruses. (a) Antigenic drift, responsible for seasonal epidemic, is a subtle change in viral glycoproteins (HA, NA) in an already circulating influenza subtype which preserves the same immune response. (b) Antigenic shift, responsible for pandemic outbreaks, consists on the introduction of completely new, segments usually from avian influenza subtype originating viruses with new glycoprotein into circulating human influenza subtype. This leads to appearance of new influenza subtype to which humans have no immunological memory to.

Type B and C influenza viruses are almost exclusively restricted to humans as a host, therefore influenza A virus is the perfect candidate of possible introduction of a new viral subtype in the human population *(Neumann, Noda et al. 2009)* with viral antigen to which most humans have no immune memory to *(Neumann and Kawaoka 2006)*. In IAV, 18 antigenically distinct HA and 11 NA proteins have been described so far. All possible combinations between them define the different subtypes of IAV. Once a new influenza subtype is introduced in human population, it may lead to human to human transmission that would cause pandemics, and occasionally serious infection outbreaks with severe illness and high mortality rate. Accordingly, there have been three major H1N1 influenza pandemics outbreaks in the twentieth century, and recently in 2009 the first pandemic of the 21st century.

The pandemic of 1918-1920 “Spanish” flu was the most devastating of all of them. Although the severity of infection led to death in 20-50 million people it is believed that this number is underestimated since there is a limitation of data, non-registration, misdiagnosis etc. *(Johnson and Mueller 2002)*. “Spanish” flu pandemic showed an unusual mortality pattern with high mortality rates in healthy young adults (age 15 to 34) and children. Genetic characterization of this virus several decades later showed that it belongs to H1N1 influenza A subtype *(Taubenberger,*

Reid et al. 1997) which along with secondary bacterial infection *(Walters, D'Agnillo et al. 2016)* caused high mortality. Studies have shown that viral replication complex, nonstructural protein 1 (NS1) and surface protein hemagglutinin (HA) contributed to its virulence *(Neumann, Noda et al. 2009).* Reconstituted 1918 influenza virus showed high virulence and mortality in murine model, embryonated chicken eggs *(Tumpey, Basler et al. 2005)* and macaque model (primate) as well as deregulated antiviral response that caused insufficient immune response that led to fatal outcome *(Kobasa, Jones et al. 2007)*.

The H1N1 IAV that appeared in 1918 circulated and caused occasional season epidemics in human population and pigs *(Logan and Mac 1951)* until its complete disappearance in 1957 when it was replaced with new human/avian reassortant H2N2 and H3N2 viruses *(Scholtissek, Rohde et al. 1978)*. The "Asian" flu emerged in Southern China in 1957. During 1957 and 1958, human/avian reassortant H2N2 virus caused excessed mortality worldwide causing almost 70 000 casualties in USA only *(CDC)*. A new reassortant H3N2 ("Hong Kong" flu) emerged in July 1968 and replaced the H2N2 virus. Unlike the previous pandemics, severe illness and high mortality rates were observed in the elderly (above 65 years of age) with 33 800 registered deaths in 1968-1970 in USA. "Hong Kong" flu therefore is considered to be the mildest pandemic of the 20th century *(CDC)*.

An H1N1 virus re-emerged again in 1977, "Russian" influenza, and resembled to that circulating in the 1950s *(Nakajima, Desselberger et al. 1978)*. Both H1N1 and H3N2 viruses have been co-circulating in human population to this date occasionally generating novel hybrid H1N2 viruses *(Guo, Xu et al. 1992)*. The most recent pandemic was originated in spring of 2009 when a new swine-origin H1N1 influenza A virus capable of human to human transmission emerged in USA and Mexico *(CDC 2009-2010)*. Genetic studies of this new A(H1N1)pdm09 virus showed that it is a triple reassortant derived from North American H3N2, swine H1N1 and Eurasian "avian-like" swine H1N1 viruses *(Girard, Tam et al. 2010)*.

The actual number of cases worldwide during 2009 pandemic remains unknown. It has been estimated that about 60 million people had been infected with A(H1N1)pdm09 only in USA *(Bautista, Chotpitayasunondh et al. 2010)*. The most affected was younger population, children under 4 years of age and adults 18-64 of age. Unlike seasonal influenza, a high portion of hospitalization and deaths due to AH1N1pdm09 infection occurred in non-elderly adults *(Presanis, De Angelis et al. 2009)*. With only 18500 laboratory confirmed deaths it has been estimated that pandemic H1N1 2009 produced between 151700 to 575400 fatalities due to both respiratory and cardiovascular complications, 80% of them in adults under 65 years of age and 51% occurred in Southeast Asia and Africa *(Dawood, Iuliano et al. 2012)*.

In addition to occasional pandemics, other influenza virus subtypes (swine, avian) can be transmitted from animals to humans (avian A/H5N1 in 1997, Hong Kong) causing severe infection. Furthermore, H5 and H7 subtypes constitute highly pathogenic avian influenza viruses (HPAIV), with mortality rate in humans of almost 100% *(Alexander 2007)*. An effective human to human transmission of these highly pathogenic viruses poses a constant concern about the possibility of future human pandemic *(de Jong, Claas et al. 1997)*. HPAIV H5N1 "bird" flu spread in birds causing severe outbreaks in poultry from Southern China to all East Asia, Central Asia, Europe and Africa during 2004-2006. This virus caused occasional viral infections to humans who were in direct contact with infected animals. Although, unable for human to human transmission, H5N1 virus caused severe illness with high mortality rates in humans. Avian A/H7N7

strain which appeared in Netherlands in 2003, with 450 infected people showed no human to human transmission but increased pathogenicity than seasonal strains *(Koopmans, Wilbrink et al. 2004)*. Sometimes human to human transmittable reassortant strains do not show increased pathogenicity such was 1999 avian A/H9N2 which appeared in Hong Kong displaying mild symptoms in patients, but showed some genome similarity to highly pathogenic H5N1 isolates few years before *(Lin, Shaw et al. 2000)*. Therefore, human to human transmittable and highly pathogenic reassortant strain is a rare phenomenon having into account the huge potential of influenza virus to evolve and adapt to new host. However, it does present a constant threat and risk to public health, since avian subtypes (H5N1, H7N7 and H9N2), to which humans are completely immunologically naïve, circulate in domestic and wild poultry and can be easily introduced in human population causing serious infections with severe illness and threatening medical conditions. More recently in 2013 avian origin H7N9 virus infected several persons in Shanghai, China with high mortality rate and rapid lower respiratory tract infection. Complications included respiratory distress syndrome and multi-organ failure *(Gao, Cao et al. 2013)*.

All these outbreaks present a constant threat to human health and great economic challenge especially in the less developed countries where monitoring of existing influenza reservoirs in both domestic and wild poultry is not sufficient or adequate. Therefore, a detailed insight into pathways by which these viruses evolve and become lethal is the key to develop more effective prevention and protection strategies against highly pathogenic and transmittable influenza strains.

2. Structure of influenza A virus

Since the first isolation of infectious particles in both pigs *(Shope 1931)* and human *(Wilson Smith 1933)* in the early 1930s, numerous studies have revealed the structure of influenza A viral particle. It possesses a segmented negative single stranded RNA (ssRNA) genome. Influenza A virus genome is formed out of 8 ssRNAs that encode 16 proteins, including a new one detected very recently *(Yamayoshi, Watanabe et al. 2015)*.

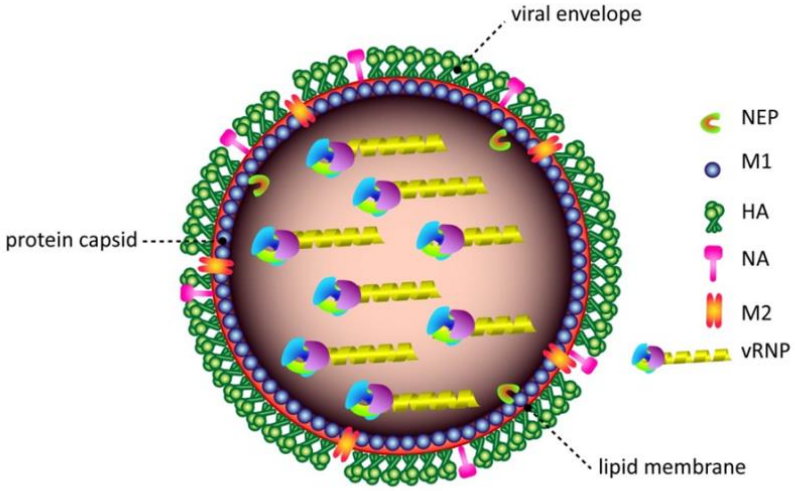


Figure 2. Structure of H1N1 viral particle. Viral envelope is consisted of hemagglutinin (HA), neuraminidase (NA) and matrix protein 2 (M2) situated in the lipid membrane positioned just above protein capsid formed by the matrix protein 1 (M1), which interacts with M2 on the outer, and nuclear export protein (NEP) on the inner side of particle. Nucleus of viral particle contains 8 viral ribonucleoproteins (vRNPs).

The surface of the viral particle contains a lipid membrane originated from the cellular membrane of infected host cell. Two glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) together with the matrix protein 2 (M2) embedded in the surface of the viral particle, are the primary target for neutralizing antibodies during the infection. There are 18 antigenically distinct HA and 11 NA proteins described so far. All possible combinations between them define the different subtypes of influenza A virus. Some aquatic wild birds (ducks, geese, swans etc.) are the natural reservoirs of all characterized subtypes of influenza virus. In bats only, H17N10 *(Tong, Li et al. 2012)* and H18N11 *(Tong, Zhu et al. 2013)* subtypes have been described. Matrix protein 1 (M1) forms an inner layer of viral particle, and interacts with both M2 on the outer side and NEP on the inner side of the particle. In the nucleus of viral particle, 8 viral ribonucleoproteins (vRNPs) can be observed *(Figure 2)*. vRNPs are formed out of (-) ssRNA, completely coated with nucleoprotein (NP) and with viral polymerase bound to a double-helix RNA structure formed by the 5' and the 3' complementary ends of (-)ssRNA.

Viral RNA dependent RNA (vRdR) polymerase is formed out of 3 subunits: polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA) subunits. More detailed description of viral polymerase will be discussed latter on.

2.1 Hemagglutinin HA

Hemagglutinin (HA) represents the primary mediator of viral entry. It is a trimer that has two structurally distinct regions (stalk and globular regions) *(Wilson, Skehel et al. 1981)*. Viral entry depends on two independent conformational changes in the HA molecule. The first includes cleavage of HA precursor structure into HA1 and HA2. This allows the formation of a fusion competent structure that can bind to HA receptors on the cell surface and initiate viral entry. After the internalization of viral particle there is a second conformational change, a relocation of the N-terminal domain of the HA2 subunit. This change is induced by a lower pH in the cell endosome. Fusion peptide domain, which is highly conserved in influenza A and B viruses, is relocated to interact with the endosomal membrane and is the crucial part of the fusion process *(Skehel and Wiley 2000, Steinhauer 2010)*. HA glycoprotein attaches to the glycan sialic acid-containing receptors on the surface of the cell membrane. Sialic acid (SA), a derivative of neuraminic acid, is present in the termini of oligosaccharides attached to glycoproteins and glycolipids on the cell surface. Infectivity of influenza virus is influenced by sialic acid species (N-acetylneuraminic and N-glycolylneuramic acid) and the type of linkage (α2,6 or α2,3) to galactose (Gal) on the glycan oligosaccharides. Hemagglutinin of human influenza A virus recognizes SA α2,6 Gal *(Rogers and Paulson 1983)*, which is the linkage found in the epithelial cells of the human upper respiratory tract *(Couceiro, Paulson et al. 1993)*. Linkage of HA to SA α2,3 was found on the epithelial cells of duck intestine and it has been shown that avian viruses preferentially bind to these receptors, while pig trachea epithelia contains both types of SA and both types of linkages, which makes them a unique "mixing vessel" for generation of new reassortant viruses *(Ito, Couceiro et al. 1998)*. It has been documented that despite the difference in the specificity of the receptors, viruses originated from other species (avian, swine) have infected humans causing lethal infections (pandemics, local epidemics) due to SA α2,3 receptors on the ciliated cells of human respiratory tract *(Matrosovich, Matrosovich et al. 2004, Ibricevic, Pekosz et al. 2006)*. Although avian viruses can potentially infect humans with efficient human to human transmission they have to overcome receptor specificity, since they preferentially recognize SA α2,3 receptors. The ability of some avian strains to recognize SA α2,6 receptors provides efficient replication in the human upper respiratory tract and it also aids in transmission via droplets, which was the case in previous pandemics *(Matrosovich, Tuzikov et al. 2000, Gamblin, Haire et al. 2004)*.

The structure of HA protein has been the target of many studies since its change of affinity from SA α2,3 to SA α2,6 linkage may be a critical step in generating pandemic influenza strains. Over 30 years ago it has been demonstrated that difference in just one amino acid changes affinity of HA from avian to human receptors *(Rogers, Paulson et al. 1983)*. Nowadays, several of these changes have been described and identified as key factors determining the host specificity binding to HA receptors. Mutations Q226L and variable position 228 in human H2 and H3 strains increase their affinity to SA α2,6 receptor, as well as E190D, and variable position 137 and G225E in human H1 strains *(Matrosovich, Tuzikov et al. 2000, Ni, Kondrashkina et al. 2015)*. Avian HA that has both 226L/228G or 226L/228S in HA, preferentially binds to SA α2,6 receptors in the upper respiratory tract of ferrets, efficiently replicates and induces high levels of neutralizing antibodies compared to the strain with the 226Q/228G residues *(Chen, Zhou et al. 2012)*.

2.2 Neuraminidase NA

NA gene encodes neuraminidase (NA), one of the surface proteins of influenza A virus particles *(Figure 2)*. NA possesses sialidase enzyme activity, which allows the removal of sialic acid from the cell membrane liberating viral particles from cells. Similarly to HA, the neuraminidase needs to adapt to human host receptor, containing SA α2,6 linkage in order to prevent self-aggregation and obtain efficient replication. NA is a tetramer that is formed out of a box-shaped head and a slender stalk *(Varghese, Laver et al. 1983)*. The length of the stalk affects host range of influenza A virus and the longer the stalk is, the more efficient replication is in cells culture, eggs *(Zhang, Xue et al. 2011)* and murine model *(Castrucci and Kawaoka 1993)*.

2.3 Matrix proteins

M segment encodes two proteins, matrix protein 1 (M1) and matrix protein 2 (M2). M1 derives from the translation of the collinear transcript of M segment and M2 derives from the spliced form of the M segment transcript. An alternative spliced form of this segment which encodes M3 peptide has been described *(Shih, Nemeroff et al. 1995)* but its function seems to be unessential for viral growth in cell culture *(Jackson and Lamb 2008)*. Another alternative spliced form of M segment, mRNA4 encodes an M2-related protein, M42 *(Wise, Hutchinson et al. 2012)*. M1 protein is a dimer and forms matrix protein capsid based just beneath the lipid envelope.

M1 interacts with viral ribonucleoproteins on the inner side of the particle, and with HA, NA and M2 proteins on the surface, and also establishes interactions with nuclear export protein (NEP) *(Yasuda, Nakada et al. 1993)* *(Figure 2)*. This makes it a unique bridge between envelope and core of the viral particle *(Nayak, Hui et al. 2004)* with a major role in budding and formation of newly synthesized virions *(Gomez-Puertas, Albo et al. 2000)*, and in the nuclear transport of the vRNPs in infected cell *(Martin and Helenius 1991)*.

M2 membrane protein has three different domains: extracellular domain that is a target for the host immune response *(Lamb, Zebedee et al. 1985)*, transmembrane domain that has ion channel activity and is involved in uncoating of viral particles *(Pinto, Holsinger et al. 1992)* and intracellular domain that interacts with M1 protein *(Zebedee and Lamb 1989)*. Since M1 and M2 proteins are involved in various processes during viral infection, M segment is much conserved in influenza A strains. This makes the M segment host specific; one of many factors that determinate host range of influenza A viruses. The M2 has a stronger selective pressure than M1 in both human and swine strains since it is a major antigen marker *(Furuse, Suzuki et al. 2009)*.

2.4 Nucleoprotein NP

Nucleoprotein (NP) is a constituent of viral ribonucleoproteins vRNPs. Viral RNA segments are coated with strings of NP protein. Each NP binds viral RNA at every 24 nt in order to form ribonucleoprotein complexes (vRNPs) *(Ortega, Martin-Benito et al. 2000)*. RNPs complexes are located inside the particle core and can be described as double-helical RNP structures *(Ye 2010)*. NP is multifunctional protein important in various viral processes: intracellular viral genome trafficking, replication, packing and assembly of virions. It is phylogenetically conserved among influenza A strains.

This protein has head and body domains with an RNA binding domain in the groove between these two domains *(Coloma, Valpuesta et al. 2009)*.

2.5 Non-structural 1 (NS1) and nuclear export (NEP) proteins

NS1 derives from the translation of the collinear transcript of NS segment while NEP derives from the spliced form of the NS segment transcript. An alternative spliced form of this segment which encodes NS3 peptide which seems to positively affect viral growth has been described *(Selman, Dankar et al. 2012)*. NS1 is a small protein with a major function in counteracting the host antiviral response and constitutes one of the main virulence factors of influenza virus. It is composed of two functional domains the N-terminal RNA binding domain and the C-terminal effector domain. This multifunctional dimeric protein binds to dsRNA in infected cells before they are detected by the cellular dsRNA viral receptors. By doing so, it inhibits the activation of cellular antiviral response and delays the IFN-mediated response *(Garcia-Sastre, Egorov et al. 1998)*.

NEP is a very small protein that has a key role in the nuclear export trafficking of vRNPs *(Iwatsuki-Horimoto, Horimoto et al. 2004)*. NEP enters the nucleus of the infected cells by passive transport but its export depends on active transport trough CMP-1. Viral strains of influenza A virus with deficient NEP have a delayed nuclear export of vRNPs which result in decreased viral growth in both cell culture and mice *(Iwatsuki-Horimoto, Horimoto et al. 2004)*.

2.6 Viral RNA dependent RNA polymerase (vRdRP)

Infection and spreading of virus mostly depend on two crucial processes, transcription and replication of viral genome. Both of these processes take place in the nucleus of the infected cell. Viral RNA dependent RNA polymerase (vRdRP) has a central role in the life cycle of influenza A virus. Viral polymerase is a heterotrimeric complex formed by three subunits; polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA).

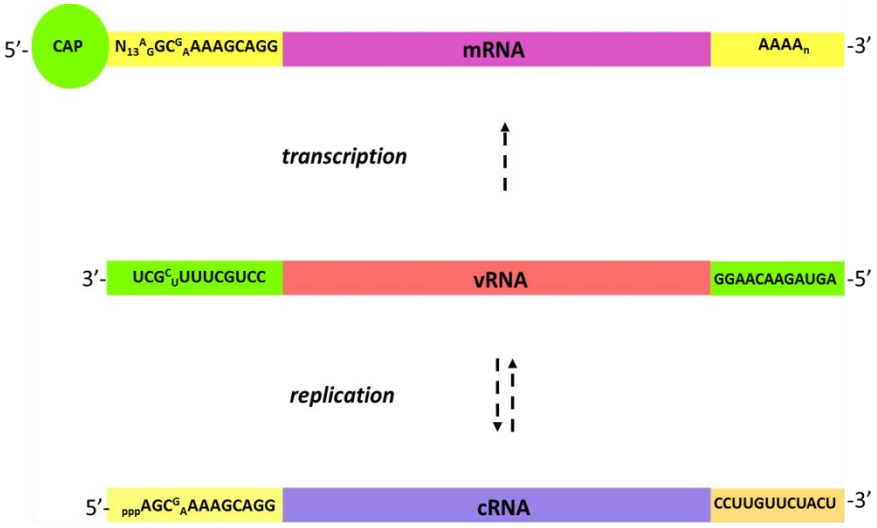


Figure 3. Transcription and replication of viral segments during the infection. Viral RNA is transcribed (a) to mRNA with CAP structure at 5'end and replicated (b) to complementary RNA (cRNA) by polymerase, which then serves as a template for accumulation of viral genome

All vRNA segments possess 3' and 5' highly conserved ends that have partial complementary sequences which form a doble-helix vRNA panhandle structure. Polymerase complex binds to the promoter situated at the panhandle structure on each viral segment *(Fodor, Pritlove et al. 1994)* and its recognition by the viral polymerase is crucial for initiation of both transcription (vRNA to mRNA) and replication (vRNA to cRNA as well as cRNA to vRNA) of the viral genome *(Figure 3)*. PB1 polymerase subunit contains the conserved motifs that form the polymerase active site *(Poch, Sauvaget et al. 1989)*. Transcription begins with a cap-snatching process in which methylated *cap* structure of host pre-mRNA 5' end is first recognized and bound by the PB2 subunit *(Guilligay, Tarendeau et al. 2008)* and then cleaved by a PA subunit endonuclease activity, 10-13 nucleotides away from the cap structure *(Dias, Bouvier et al. 2009)*. This snatched *cap*–oligo is then used as a primer for the viral mRNA synthesis *(Figure 3a)*. Recent reports show that the 'cap-snatching' process requires substantial conformational reorganization of the complex, especially PB2 subunit *(Reich, Guilligay et al. 2014)*. Viral mRNAs possess a poly A tail at 3' end generated by the stuttering mechanism of the polymerase complex *(Reich, Guilligay et al. 2014)*.

Replication proceeds in a primer-independent manner *(Figure 3b)*. Viral RNA replication involves the synthesis of cap-independent, full-length positive-stranded RNAs complementary to the genomic vRNAs, named cRNAs, which serve as a template for the amplification of new vRNAs. Both vRNAs and cRNAs are coated by NP forming cRNPs and vRNPs, respectively. A recent study suggests that replication may be initiated by binding of the 5' hook to a pocket of the PB1 and PA interaction *(Pflug, Guilligay et al. 2014)*. The mechanism by which polymerase is able to switch from one process to another is yet to be fully described.

3. Polymerase subunits and pathogenicity of virus

Pathogenicity is a multifactorial property of every influenza A viral strain. It depends on the nature of the virus itself, the immune response of the host and its capacity to overthrow the infection in a complex interplay of both factors. Changes in genome allow virus to adapt to new conditions and viral polymerase has a key role in the process of generation of new mutations in every viral gene. Besides frequent changes of the surface proteins (HA, NA), which enable infection, adaptive mutations are found in every viral gene responsible for modulation of host antiviral response or efficient replication and growth of virus in the host. Influenza A virus polymerase generates one spontaneous mutation per replicated genome *(Drake 1993)*. Without proofreading activity the viral polymerase is capable of producing 10^4 mutated viruses in just one infected cell *(Boivin, Cusack et al. 2010)*. This feature of polymerase gives a huge variability of viral genome in just one replication cycle. It permits a virus to evolve very soon, very fast and obtain resistance basically to all therapeutic tools used so far.

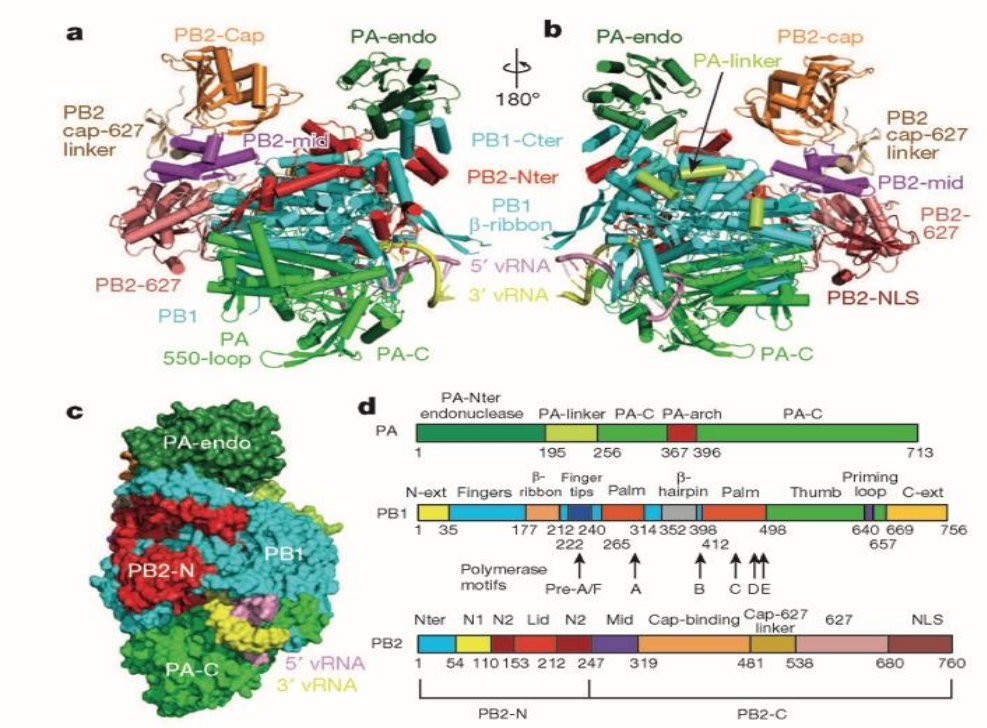


Figure 4. Influenza A virus polymerase. Viral polymerase complex presented in two different views (a) and (b) with vRNA promotor (pink and yellow) following the color code presented in figure (d) showing the subdomains of every polymerase subunit; (c) monomeric structure of viral polymerase and its subunits in space filling side-view representation (**10.1038/nature14008**)

3.1 Polymerase basic 2 subunit PB2

Protein polymerase basic 2 subunit (PB2) is transcribed from the longest viral segment. N-terminus of PB2 protein interacts with C terminus of PB1 in the polymerase complex *(Sugiyama, Obayashi et al. 2009)*. This protein has a 5'-cap structure binding activity *(Figure 4)* that is used for binding to host pre-mRNAs, a crucial step for viral transcription *(Poole, Medcalf et al. 2007)*. PB2 protein plays an important role in the pathogenicity of influenza virus. It contains a host range determinant at position 627 since only one amino acid change (E→K) at this position in PB2 allows the growth of an avian virus in mammals. Most avian strains have a glutamic acid at this position, while almost all human strains possess a lysine *(Subbarao, London et al. 1993)*. Avian H5N1 strains containing a lysine at this position have increased pathogenicity and infectivity in mice *(Hatta, Gao et al. 2001)*. This leads to the conclusion that 627K in PB2 is a pathogenicity marker in mammalian species. The temperature on which different origin strains are replicated provides a possible explanation for this phenotype. Human strains replicate in upper respiratory tract at 33˚C while avian in intestine at 41˚C. Avian 627E strains in PB2 show thermosensitive phenotype with low efficiency of replication at lower temperature, while human 627K strains had the same rate of replication *(Massin, van der Werf et al. 2001)*.

Another amino acid has been characterized as host range determinant involved in adaptation of H5N1 strain to mice *(Li, Chen et al. 2005)*. Adaptive change D→N in PB2 protein at position 701 is responsible for the enhanced binding to nuclear transporter importin α1 in mammalian cells while it does not affect PB2 nuclear transport in avian cells *(Gabriel, Herwig et al. 2008)*. The 701 change can be accompanied by another nearby, S→R, change at position 714, which has been found in the avian strains highly pathogenic for mammalian species. This mutation promotes the

phenotype described by 701 change; accumulation of importin α in nucleus of infected cell and enhanced nuclear transport of PB2 polymerase subunits *(Czudai-Matwich, Otte et al. 2014)*. The 588 change (T→I) found in the pandemic 2009 strain (A(H1N1)pdm09) showed to be an adaptive change to mammalian cell replication and increased virulence and mortality in mice *(Zhao, Yi et al. 2014)*. PB2 588 (T→I) proteins have lower binding affinity for mitochondrial antiviral signaling proteins (MAVS) and therefore inhibit induction of interferon type I antiviral response *(Zhao, Yi et al. 2014)*.

Recently, an alternative splicing product of PB2 segment has been detected in infected cells with several H1N1 strains. Similar donor-acceptor splice sites were found in all H1N1 strains prior to A(H1N1)pdm09; however H3N2 and pandemics H1N1 strains do not possess these sequences *(Yamayoshi, Watanabe et al. 2015)*. Protein encoded by this spliced RNA, PB2-S1 protein, localizes with mitochondria and inhibits RIG-I pathway thus alters antiviral response in infected cells *(Yamayoshi, Watanabe et al. 2015)*.

Another important domain of PB2 protein is its mitochondrial antiviral signaling proteins (MAVS) binding domain. Unlike avian strains, PB2 of human influenza strains co-localizes with MAVS and induces lower levels of antiviral response mediated by interferon β (IFN-β) in cell culture while viral growth is not affected *(Graef, Vreede et al. 2010)*. These strains are attenuated in murine model. It has been shown that N→D change at position 9 in avian strains is responsible for acquiring functional MAVS binding domain *(Graef, Vreede et al. 2010)*. Swine origin 2009 pandemic strains do not possess this change in PB2 protein *(Graef, Vreede et al. 2010)*.

3.2 Polymerase basic 1 subunit PB1

PB1 segment encodes two proteins, polymerase basic 1 (PB1) and PB1-F2 protein which is the product of a +1 displacement in the reading frame. PB1 protein interacts both with PB2 and PA subunits of the viral polymerase and contains the polymerase active site, with sequence motifs characteristic of viral RNA-dependent RNA polymerases which are essential for its activity *(Figure 4)*. A N375S change has been found in all pandemic strains before 2009. Interestingly, although human strains almost uniformly possess serine, avian strains show much more diversity having serine, asparagine and threonine at this position *(Taubenberger, Reid et al. 2005)*.

PB1-F2 is recognized by the CD8+T lymphocytes *(Chen, Calvo et al. 2001)* and exposure of monocytes to PB1-F2 led to induced apoptosis and cell death *(Chen, Calvo et al. 2001)*. In the absence of PB1-F2, PB1 has altered location, which affects polymerase activity, indicating that interaction between PB1 and PB1-F2 regulates the polymerase activity *(Mazur, Anhlan et al. 2008)*. Changes in this protein lead to higher pathogenicity and lethality in mice model while delaying the adequate innate immune response *(Conenello, Zamarin et al. 2007)*. PB1-F2 was not present in A(H1N1)pdm09 virus.

3.3 Polymerase acidic subunit PA

Polymerase acidic (PA) and PA-X are proteins transcribed from the PA viral segment. PA contains two domains: N-terminal (1-256 aa) and C-terminal (257-716 aa) separated by a peptide linker *(Maier, Kashiwagi et al. 2008)*. The C terminal domain is responsible for the interaction with N-terminal of PB1 polymerase subunit *(Obayashi, Yoshida et al.*

2008). Aside from stabilizing the polymerase complex, PA subunit is involved in proteolysis of both viral and host proteins *(Sanz-Ezquerro, Zurcher et al. 1996, Rodriguez, Perez-Gonzalez et al. 2007)*. Cristal structure of PA subunit gave us a detailed insight into both, the C and N-terminal domains *(Figure 4)*, demonstrating that N-terminal domain contains an endonuclease putative active site motif $P_{107}D_{108}X_{10}E_{119}K_{134}$ involved in the cap-snatching process of viral transcription *(Yuan, Bartlam et al. 2009)*. Several changes in the PA protein have been described as host range determinants. For instance, 2009 pandemic strains have an avian PA subunit with various adaptive changes to mammalian host such are at 85(T→I), 186(G→S) and 336(L→M) positions that enhance viral growth in cell culture. The T85I change provides high titers in human alveolar epithelial cells A549 while L336M change increases viral pathogenicity in mice *model (Bussey, Desmet et al. 2011)*.

PA-X is produced through a ribosomal frameshifting and contains the endonuclease domain of the PA polymerase subunit followed by a new sequence of 61 aa translated from a different reading frame. It is involved in various processes in the viral infection by modulating the host innate antiviral response *(Jagger, Wise et al. 2012)*. PA-X induces cellular shut off in the cell culture more efficiently than PA N-terminal fragments *(Desmet, Bussey et al. 2013)*. Sequence of PA-X is variable and often trunked in H3N2 and H3N8 strains, which might be associated with host specific adaptation of the virus *(Shi, Jagger et al. 2012)*. PA-X was not detected in any A(H1N1)pdm09 strain.

4. Generation of defective genomes (DGs)

Generation of defective viruses is a unique feature of some RNA viruses (dengue virus, Sendai, hepatitis, influenza…). In the early 50s, it was observed accumulation of defective and non-infectious viruses in serial passages of influenza viruses at high multiplicity of infections in embryonated chicken eggs *(Von Magnus 1954)*. Defective interfering RNAs (DIs) were detected and named later when the suitable technics for their detection were available. They possess the 3' and 5' ends of the parental RNA segments and most have large, single, central deletion *(Figure 5)*. They are capable of interfering with viral replication by competing with the initial full-length viral genes *in vitro (Huang 1973)*. These incomplete genome RNAs may not always interfere with viral replication if they are not accumulated at high concentration compared to complete RNA genome, but other attributes of this short RNAs have been described. Therefore, the correct form to name them is defective genomes or DGs (further text). Since their accumulation may disturb replication and consequently viral spread, the ratio between generated DGs and full-length RNA segments may determine the final outcome of infection.

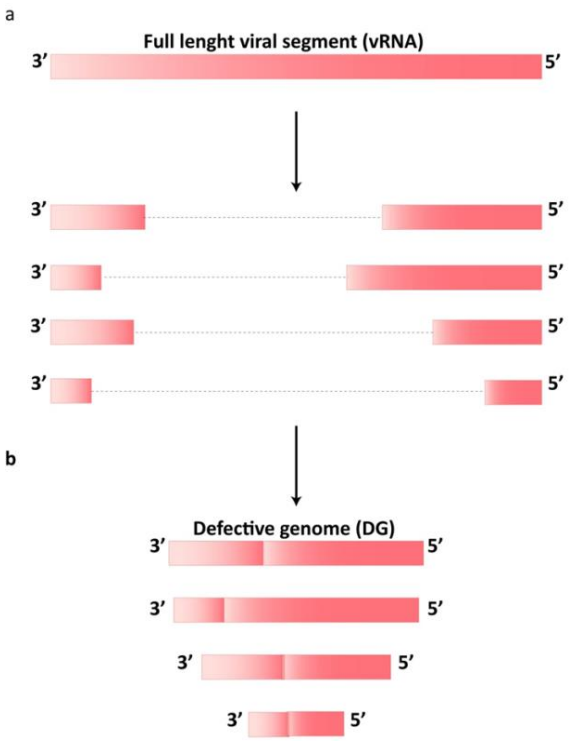


Figure 5. Defective genome (DG) accumulation during replication. (a) Viral segments during the process of replication loss a large central fragments which ends in accumulation of deleted products (b), small RNAs with identical 3′ vRNA and 5′ vRNA ends and incomplete viral genome information (DGs).

High production of DGs would, at one point, work as a limiting factor for viral propagation. It has been described that the majority of DGs detected in influenza virus is originated from polymerase segments PB2, PB1 and PA *(Dimmock and Easton 2014)*. Most DGs are 180-1000 nt long *(Davis and Nayak 1979, Noble and Dimmock 1995)*, and have about 20% of original length in average (400 nt) *(Dimmock and Easton 2014)*.

Defective particles are unable to replicate without the presence of infective particles with complete segment information *(Nayak 1980)*. The mechanism of production of DGs is still unclear and even though tremendous efforts to study this process have been employed in the past few decades, generation of defective genomes in influenza virus infections remains an unknown process *(Figure 5 and 6)*. DGs are also generated *in vivo*; sequence analysis of nasopharyngeal samples from patients infected with A(H1N1)pdm09 strains showed similar distribution of DGs, generated principally from polymerase genes with similar length found in *in vitro* samples. Interestingly enough, patients that had been in direct contact shared the same DGs *(Saira, Lin et al. 2013)*.

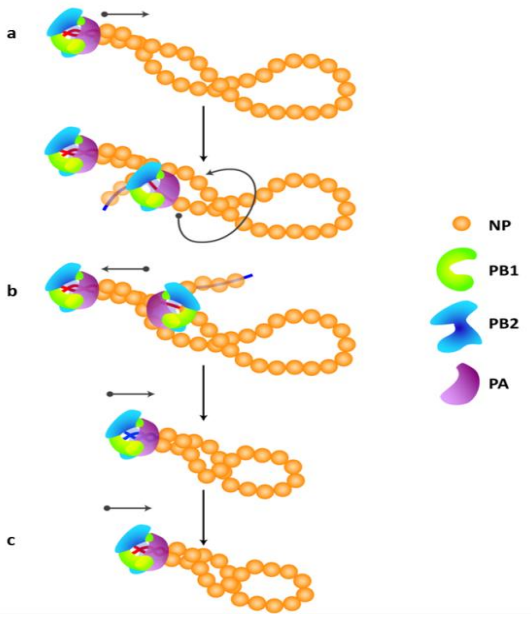


Figure 6. Possible mechanism of generation of defective genomes during viral replication process. (a) Viral polymerase complex (PB1, PB2 and PA) starts replication of viral genome (*red*) by removing the nucleoprotein (NP) from vRNPs, in 5'-3' sense producing complementary (+) sense RNA stains (*blue*). During this process viral polymerase may flip from 3'-end side and proceeds replication of 5'-end side of viral genome (b), producing (+) strain, cRNA, which possess complementary 3' and 5'ends of (-) viral genome and large central deletion. (c) Replication of this short, cRNA into viral genome, vRNA (*red*) leads to progressive accumulation of viral genome with uncomplete genome information, defective genome (DG). Other alternative of DGs production may occur during the replication process from cRNA to vRNA.

All experiments with DGs particles are not fully reproducible. Naturally generated stocks possess mix of DGs with different interference capability and its content is not stable throughout passages. Cloned viruses that have single main type of DG RNA provide a protection in secondary influenza virus infection in both mice *(Dimmock, Rainsford et al. 2008)* and ferrets *(Dimmock, Dove et al. 2012)*, giving the possibility of using DGs as a tool against influenza infections.

4.1 Effects of DGs during the infection

Mammalian cells have developed various strategies in order to detect antigens of different pathogens. Pathogen recognition receptors (PRRs) recognize antigens and activate the cellular response in infected cell. Antiviral response of infected cells depends on the adequate and quick recognition of viral antigens by cells' PRRs. It has been observed that short dsRNA or ssRNA activate antiviral response predominantly by binding to one of cytoplasmic PRRs that recognizes these molecules with high affinity (RIG-I). Induction of this antiviral pattern leads to induction of type I interferon response *(Kell and Gale 2015)*. Detailed antiviral response will be the subject of the next chapter.

Truncated forms of viral genome (DG) can, besides the already described interference in replication of viral genome, induce the antiviral response in infected cells. Defective genomes have been detected in various plant and mammalian cell cultures and also in patients with acute and chronic hepatitis A *(Nuesch, de Chastonay et al. 1989)*, B *(Rosmorduc, Petit et al. 1995)* and C *(Noppornpanth, Smits et al. 2007)* infection, HIV infections *(Inoue, Hoxie et al. 1991)*, Dengue virus *(Li, Lott et al. 2011)* and influenza virus *(Saira, Lin et al. 2013)* infections. Copy-back defective genome in Sendai

virus in *in vitro* infection induces cytokine response and maturation of mouse and human dendritic cells, which may provoke earlier induction of adaptive immune response *(Mercado-Lopez, Cotter et al. 2013)*. Sendai virus with high loads of defective genomes promotes expression of interferon stimulated genes (ISGs), independently of TLR or type I interferon pathway *(Yount, Gitlin et al. 2008)*. In *in vivo* infection with the same virus it has been shown induction of IFN-β and IL-6 in the lungs of infected mice *(Tapia, Kim et al. 2013)*. Furthermore, infection of human lung slices with respiratory syncytial virus (RSV) with high DGs content provokes a vigorous antiviral response dominated by IFN-λ1 and IFN-β stimuli *(Sun, Jain et al. 2015)*. Similar results were observed in influenza A virus infected mice. Antiviral response in animals infected with a virus generating large amount of DGs, induced high expression of IFN-β, with reduced morbidity rate of mice infected with influenza A H1N1 virus *(Tapia, Kim et al. 2013)*. Therefore, in both *in vivo* and *in vitro* infections, antiviral response with DG enriched viruses is stronger than in infections with viruses with low DG content independently of viral origin or mechanism of DG generation.

Infection of mice without activated adaptive immune system showed that DGs are able to protect animals in the early stage of infection but the clearance of infection was obstructed and delayed *(Scott, Meng et al. 2011)*. Innate immune response against viruses which accumulate low amount of DGs is not enough to protect from developing a severe illness. Therefore, adaptive immune response needs to be activated by already DG-induced active innate response in order to obtain full protection *(Scott, Meng et al. 2011)*. DGs rich viruses can both enhance maturation of dendritic cells as well as the adaptive immune response *(Yount, Kraus et al. 2006)*. DGs have a universal potential to induce antiviral response in infected cells and therefore have a significant impact on the progress of infection and illness.

Accumulation of defective genomes in influenza A infection depends on the multiplicity of infection but also on the nature of viral strain itself. Changes in viral polymerase subunits may be responsible for DGs production ability in different viral strains. Thus, fidelity and processivity of viral polymerase has crucial effect on overall DGs production in influenza virus infection.

DGs upon cell entry can be transcribed into functional mRNA that can be translated into polypeptides *(Akkina, Chambers et al. 1984)*. Newly detected protein, encoded from the mRNA derived from a PB2 segment DG of H5N1 virus, alters IFN type I response in cell culture independently of the RIG-I pathway, the usual antiviral response inducted by DGs *(Boergeling, Rozhdestvensky et al. 2015)*. Unlike PB2 protein, DG mRNA product, $PB2_{\Delta}$, induces the production of IFN-β and IFN-β stimulated genes and lowers the viral titer of influenza A virus *in vitro* by binding to a MAVS on the mitochondrial membrane. In mice model, the strain with $PB2_{\Delta}$ expressed protein showed increased pathogenicity *in vivo* and enhanced cytokine response *(Boergeling, Rozhdestvensky et al. 2015)*. This opens a completely new perspective on the DGs and their expression in influenza A viruses infection.

After years and years of investigation it seems that, even though DGs are considered a byproduct of replication, there are many questions to be responded regarding their generation and functions. Other byproducts of replication, numerous mutations in viral genome, together contribute to viral pathogenicity and adaptation to mammalian cells. Different evolution strategies, where the error prone activity of viral polymerase is involved, affect humans and could be responsible for developing a severe illness and a fatal outcome.

5. Characterization of DGs production in isolates of influenza A viruses from 2009 pandemic

Despite all described data related to DGs in cell culture and animal models, the possible correlation of DGs and pathogenicity in influenza infected patients have never been evaluated.

In order to evaluate whether influenza strains with increased virulence may coexist among circulating viruses previous studies in our laboratory characterized two IAV strains from AH1N1 2009 pandemic (A(H1N1)pdm09), one isolated from a fatal case in a person with no known comorbidities (F) and the other from a patient with mild symptoms (M) *(Rodriguez, Falcon et al. 2013)*. This study showed that F virus replicates faster in cell culture and has increased pathogenicity in animal model causing significant more weight loss and mortality of animals compared to M strain *(Rodriguez, Falcon et al. 2013)*(Figure 7).

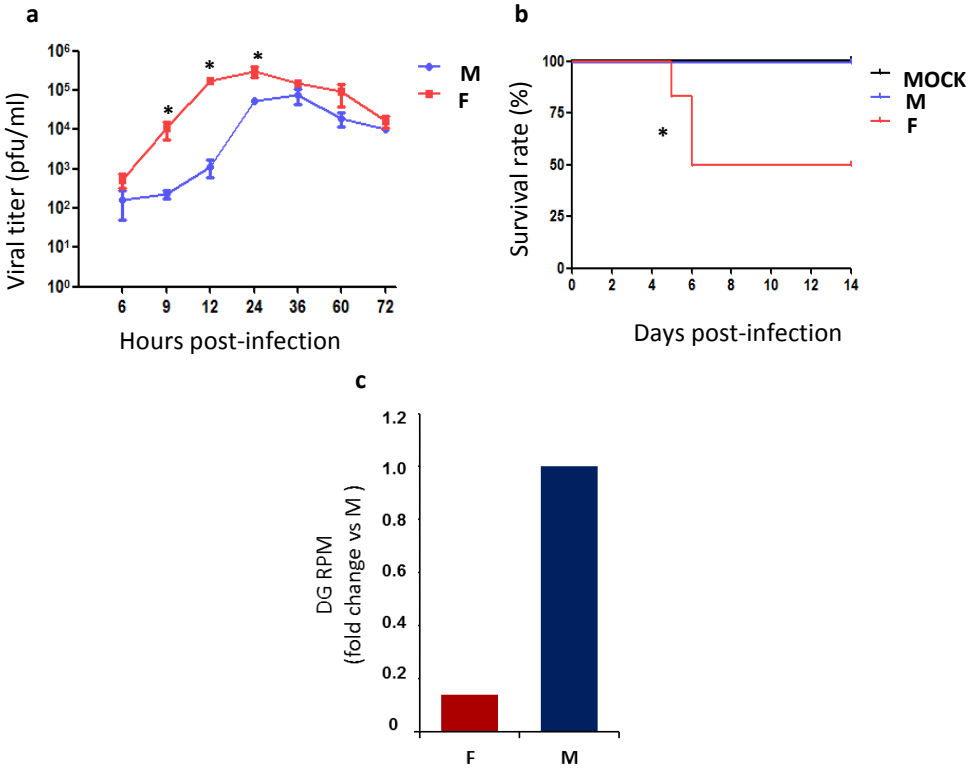


Figure 7. Virulence and pathogenicity of F clinical isolate and defective genomes produced during influenza virus infection. (a) Growth kinetic of M and F viruses in human alveolar A549 cell line infected at moi 10^{-3}pfu/cell. Viral yield was determined by plaque assay in the MDCK cells. (b) Mice were intranasally inoculated with 10^6 PFU (50 µl) of either M or F influenza viruses or were mock infected with 50 µl of PBS and were monitored daily for survival for 14 days. (c) DG ratios, calculated as jumping reads per million (RPM) that align the viral genome, determined in purified virions from the fatal (F) or the mild case (M) of IAV infection

Deep-sequencing of viral genome of these strains showed that there are 9 amino-acid changes between F and M strains in PB2, PA, NP, HA and NA gene segments. Sequence comparison with a consensus amino acid sequence obtained using the influenza virus resource database from NCBI and including around one thousand 2009 pandemic viruses isolated in the time frame of one month before and after the isolation date of M and F viruses, highlighted three amino-acid changes PB2 A221T, PA D529N and HA S127L in the F isolate *(Rodriguez, Falcon et al. 2013)*, that might be responsible for the increased virulence of F strain. In addition to the consensus viral genome, the deep

sequence of RNA isolated from F and M virions showed the presence of defective genome (DGs) in both samples, but an almost 10-fold lower DGs amount was observed in the F than in the M virus *(Figure 7)*.

6. Antiviral response in infected cells

In order to infect, the virus must first enter through nasal or oral cavities. After reaching respiratory tract, viral particles must penetrate a mucus layer that covers respiratory epithelia. Only then, virus is able to bind to pneumocytes and non-target immune cells in the respiratory tract. These cells represent the first active barrier against the virus. As mentioned before, mammalian cells have co-evolved, along with viruses, in order to quickly and efficiently recognize and neutralize viral antigens. Pathogen associated molecular patterns PAMPs, viral antigens, are recognized by cellular PRRs that are represented by distinct classes: Toll-like receptors (TLRs), retinoic-acid inducible gene I-like receptors (RLRs), NOD-like receptor family member, NOD-LRR-and pyrin domain-containing 3 (NLRP3) and C-type lectin receptors (CLPs). These receptors have a different location in the cell and represent unique guardians with a mission to eradicate infection and to alert neighboring cells and circulating specific immune cells. RLRs and TLRs are the most characterized PRRs involved in antiviral response upon influenza virus infection.

6.1 Recognition of PAMPs. First cascade of antiviral response

6.1.1 RLRs. Retinoic-acid inducible gene I - RIG-I

RLRs include RIG-I, melanoma differentiation associated factor 5 (MDA5) and LGP2 protein, which belong to the DExD/H box RNA helicases family *(Loo and Gale 2011)*. In the cytosol of the infected cell RLRs recognize 5'-trisphosphate RNAs and dsRNA which triggers a type I interferon (IFN) antiviral response *(Hornung, Ellegast et al. 2006)*. Besides 5'-trisphosphate another feature of viral RNA is necessary for RIG-I recognition, the 3'-polyU motifs of viral genome *(Saito, Owen et al. 2008)*. Through this mechanism RIG-I specifically recognizes non-self, viral RNA, avoiding the recognition of cellular RNAs *(Saito, Owen et al. 2008)*. Long dsRNA fragments are primary target of MDA5 while RIG-I shows preference to short dsRNA PAMPs *(Kato, Takeuchi et al. 2008).* More in detail, viral RNA is recognized by RIG-I helicase domain, while at the same time RIG-I caspase recruiting domains (CARD-like domains) *(Yoneyama, Kikuchi et al. 2004)* are subjected to conformational change by which these domains become exposed and can interact with tripartite motif protein (TRIM25) that binds to one of these domains and subsequently ubiquitinates the other CARD domain *(Gack, Shin et al. 2007, Gack, Kirchhofer et al. 2008)*. This facilitates CARD-CARD interaction to interferon-β promoter stimulatory 1 protein (IPS-1) also known as MAVS, VISA or Cardif. This last factor activates phosphorylation of interferon regulatory factor 3 (IRF3), IRF7 and NF-κβ and subsequently induces IFN type-I synthesis *(Kawai, Takahashi et al. 2005, Meylan, Curran et al. 2005)*. A diagram of these pathways is presented further on *(Figure 8a)*.

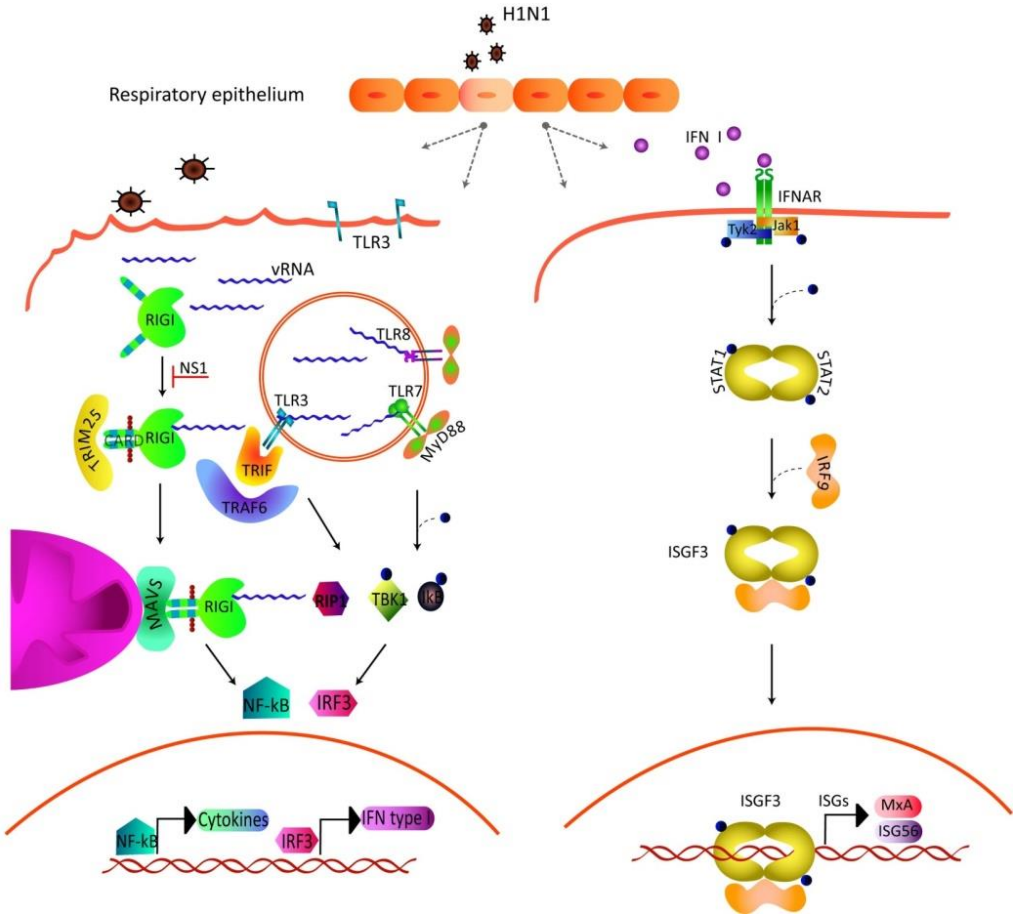


Figure 8. Antiviral cellular response. (a) Recognition of viral RNA by PRRs induces signal cascade which triggers synthesis of cytokines and IFN α and β. IFNAR receptors (IFN-α/β) on the neighboring uninfected cells (b) induces another signal cascade that ends with activation of transcription of many antiviral genes (MxA, ISG56…)

Influenza virus has adapted and evolved several mechanisms to overcome the induced antiviral cell response. One of which is trough non-structural viral protein 1 (NS1) which is capable to repress induction of IFN type I response by inhibiting antiviral RIG-I pathway *(Garcia-Sastre, Egorov et al. 1998)*. NS1 binds to TRIM25 proteins and disable ubiquitination of RIG-I CARD and downstream signalization of IFN type I response *(Gack, Albrecht et al. 2009)*.

6.1.2 Toll-like receptors - TLRs

Toll-like receptors are the best characterized family of all PRR members. TLRs are transmembrane proteins expressed on the cell surface and intracellular vesicles membrane with PAMPs recognition ability and are formed out of three domains: leucine reach repeats ectodomain, transmembrane domain, and cytosolic Toll-IL-1 receptor (TIR). During viral infections crucial TLR members sense nuclear acids; TLR3 recognizes dsRNA, TLR7 and TLR8 recognize ssRNA and TLR9, which target unmethylated CpG motifs of dsDNA. TLR2 and TLR4 can detect viral antigens but are specific for certain viruses *(Kawai and Akira 2011)*.

Most of the TLRs (among which TLR7, TLR8) are associated with MyD88 adapter and upon detection of infection, MyD88 dimerizes. This dimerization induces a signal cascade in infected cell that leads to activation of TANK binding

kinase 1 (TBK1) and inducible IκB kinase that activates IRF3 that along with IRF7 induce transcription of type I IFN in the infected cell *(Figure 8a)* *(Sharma, tenOever et al. 2003, Hacker, Redecke et al. 2006)*.

TLR3 receptor is associated with other adaptor molecule, TRIF, through its TIR domain. Activated TRIF associates with TRAF6 and TBK1 pathway *(Sato, Sugiyama et al. 2003)* that activates IRF3 *(Fitzgerald, McWhirter et al. 2003)* but also receptor interacting proteins, RIP1 and RIP3, where RIP1 is responsible for activation of NF-κβ *(Meylan, Burns et al. 2004)*. Therefore, since localization of different TLRs in virus infected cell is diverse they show different functions. TLR3 as a surface cell protein is able to detect nearby infected cells and prompts antiviral state of the uninfected cell. TLR7 and TLR8 based on the intracellular vesicles sense infection inside the cell and induce antiviral response *(Iwasaki and Pillai 2014)*.

6.2 Activation of antiviral genes. Second cascade of antiviral response

Although, RLRs and TLRs activate different signal pathways, both induce the same cell antiviral response mediated by production of interferon type I, α and β (IFN-α, IFN-β). Secreted IFN acts both autocrine and paracrine and binds to the IFN-α/β receptor (IFNAR) expressed on the surface of the cells *(Figure 8b)*. Binding of IFN to its receptor activates a cascade that starts with phosphorylation of tyrosine kinase 1 (Jak1) and 2 (Tyk2), following the phosphorylation of STAT1 and STAT2 that work as signal transducers and activators of transcription. STAT1 and STAT2 then associate with IRF9 forming an interferon stimulated gene factor 3 transcription complex (ISGF3) which is translocated to the nucleus where induces transcription of antiviral genes (ISGs) *(Goodbourn, Didcock et al. 2000)*. ISGs possess ISRE elements that are target sequence for ISGF3. Some of these genes encode antiviral proteins that affect viral production in the infected cells. Protein kinase R (PKR), 2'-5' olygoadenylate synthetase (OAS), Myxovirus resistance gene A (MxA), and IFN induced transmembrane proteins (IFITMs) are some of the proteins with antiviral effect transcribed by IFN induction.

6.2.1 ISGs

- **Protein kinase R (PKR)** upon sensing dsRNA, dimerizes and suffers autophosphorylation which leads to phosphorylation of eukaryotic initiation factor 2α-subunit (eIF2α) and subsequent inhibition of translation in the host cell *(Pham, Santa Maria et al. 2016)*.
- **2'-5' olygoadenylate synthetase (OAS)** detects dsRNA and activate RNase L which promotes RNA degradation (Kristiansen, Gad et al. 2011) and enhanced RIG-I mediated antiviral response *(Zhu, Zhang et al. 2014)*.
- **MxA** and its mouse ortholog the Mx1 protein, belong to a dynamin superfamily of large *GTPase (Haller and Kochs 2011)*. MxA is a cytosolic protein *(Ciancanelli, Abel et al. 2016)*, that inhibits nuclear transport of influenza virus NP, which affects influenza virus transcription and replication *(Kochs and Haller 1999)*. In almost all inbred mice strains Mx1 is defective and with this in mind should be analyzed all data regarding antiviral response produced in infected animals *(Cilloniz, Pantin-Jackwood et al. 2012)*.

- **Interferon inducible transmembrane proteins (IFITMs)** block viral entry but they do not show the same antiviral pattern in all viral infections. In influenza A infections IFITM3 (ISG60) response is more conspicuous than IFITM1

(ISG56) and IFTM2 (ISG54) *(Bailey, Zhong et al. 2014)*. IFITM3 is expressed in the target cells of IAV in respiratory tract, as well as in macrophages and has a fundamental role controlling influenza A virus *in vivo (Bailey, Huang et al. 2012)*.

Regardless of the pathway all these processes lead to the same aim, activation of antiviral, local, response and synthesis of type I IFN, pro-inflammatory cytokines and chemokines that are responsible for activation of innate immune response and immune cell recruitment which would subsequently lead to induction of inflammation process and viral clearance in infected tissue. Adequate host response to viral inflammation is a crucial feature in progression of illness and patient recovery process. Therefore, host antiviral and inflammation response to viral infection contribute to pathogenicity of viral strain.

7. Innate immune response upon influenza virus infection

Adequate and timely response to the influenza infection is a crucial factor in disease development and final outcome. Immune system possesses three levels of protection of pathogens, first is the physical barriers (skin, mucosae…), the second corresponds to the innate (natural) response effective 4-96 hours of infection and the third is the adaptive (acquired) response detectable after 96 hours upon the infection. Innate immune response is a non-specific and non-selective response largely dependent on the leukocyte activation, while adaptive immune response is unique and selective and therefore starts only when needed. Leukocytes detect PAMPs through PRRs (TLRs and NOD) and prompt virus clearance by induction of phagocytosis, a target cell lysis and inflammation process mediated by increased secretion of pro-inflammatory cytokines: interleukins (IL-1,6, 8, 12, 18), tumor necrosis factor-TNF, and IFN-α/β. Myeloid (granulocytes, monocytes, macrophages and dendritic cells-DC), lymphoid (T and B lymphocytes, NK, NKT cells and DC) and mast linage cell members are involved in different stages of immune response*(Mak, Jett et al. 2011)*.

Type II alveolar cells and alveolar macrophages are primary targets of influenza A virus and strong inducers of IFN mediated antiviral response. Besides antiviral proteins, infected type II alveolar cells secrete various pro-inflammatory cytokines (interleukins, IL-8 which recruits neutrophils, macrophages and mast cells *(Deshmane, Kremlev et al. 2009)* and IL-6 and chemokines (MCP-1 which recruits monocytes and macrophages, RANTES, and MIP-1β) whereas alveolar macrophages produce tumor necrosis factor α, TNF-α *(Deshmane, Kremlev et al. 2009, Wang, Oberley-Deegan et al. 2009)*. Macrophage inflammatory protein MIP-1α promotes pro-inflammatory process in cells but also inhibits proliferation of hematopoietic stem cells and has an important role controlling inflammation in influenza infection *(Cook 1996)*. The synthesis of antiviral factors that takes place during IAV infection provokes significant influx of innate immune response cells that are almost immediately involved in the virus clearance and *de novo* recruitment and activation of adaptive immune response.

7.1 Neutrophils

In inflammatory process neutrophils are the first leukocytes that target the infection focus. After detection of PAMPs, activated neutrophils immediately phagocyte the pathogens and by fuse them with phagosome scavenge the source of inflammation. Neutrophils also release the phagosome content to the extracellular matrix, which in acute and persistent inflammation can damage adjacent healthy tissue and cause acute pulmonary distress syndrome (APDS)

(Martin, Pistorese et al. 1991). Other granulocyte leukocytes, basophils and eosinophils also induce phagocytosis upon detection of infection but are represented in a smaller number in the blood than neutrophils *(Mak, Jett et al. 2011)*.

Neutrophil influx in influenza infected respiratory tissue is essential for recruitment of $CD8^+$T cells *(Lim, Hyun et al. 2015)*. Recent studies show that human neutrophils, even though they do not possess sialic acid could be infected with AH1N1pdm09 virus. Apart from entry, virus is able to replicate inside these cells and spread. It is hypothesized that these leukocytes may represent an advantage for virus spread outside the target tissue *(Zhang, Huang et al. 2015)*. Lethality by influenza A virus in the mice model has been correlated with tissue damage caused by inadequate, vigorous neutrophil influx and subsequently induced inflammation *(Brandes, Klauschen et al. 2013)*.

7.2 Monocytes

Monocytes are the prevalent cells circulating in the blood and represent 3-5% of all leukocytes. Differentiation of phagocytes to macrophages occurs in the inflamed tissue. Monocytes can also differentiate into myeloid dendritic cells (mDCs) in some physiological conditions like infections. These mDCs do not express pro-inflammatory cytokines, but chemoattractants (MCP-1, IP10), type I IFNs and thus ISGs *(Cao, Taylor et al. 2012)*.

7.3 Macrophages

Macrophages are large, long live phagocytes that patrol and reside in all organs and tissues constantly engulfing damaged host cells and cell debris as well as the pathogens. Differentiation in a particular microenvironment in their resident tissue cause subtle differences in morphology and function (alveolar macrophages (AM) in lung, Kupffer cells in liver, osteoclast in bones). Upon activation by external material (cellular debris, spent host cells, foreign entities, pathogens…) macrophages induce phagocytosis and secretion of cytokines, growth factors and chemokines alerting others leukocytes (neutrophils, T and B lymphocytes) *(Mak, Jett et al. 2011)*. AMs have an important role in viral clearance and affect virus induced morbidity. Influenza H1N1 infected mice with defected, immature AM show high morbidity and mortality despite functional and efficient adaptive response, which mark these cells as the key factor of immune protection from influenza infection *(Schneider, Nobs et al. 2014)*.

7.4 Dendritic cells

Dendritic cells (DCs) can be differentiated from both myeloid and lymphoid precursor and give numerous subtypes with subtle differences in the immune response. Along with macrophages, DCs represent professional antigen presenting cells (APC). Maturation of DCs occurs when these cells encounter pathogen which induces production of cytokines and activation of T adaptive response *(Mak, Jett et al. 2011)*. Using macaques as animal model, it has been reported that highly pathogenic H5N1 strains efficiently infect and induce apoptosis of mature DCs in both infected lung and draining nodes in the early infection, altering the synthesis of type I IFN and activation of adequate adaptive response *(Baskin, Bielefeldt-Ohmann et al. 2009)*.

Therefore, pathogenicity and virulence of any viral strain depend on a complex interplay of both viral and host factors. Viral entry, replication, activation of antiviral response in infected cell, innate immune response and ability to spread

beyond viral primary target cells greatly affect the illnesses progress and final outcome of the disease. The aim of this thesis is to analyse all these factors together in order to understand what makes an influenza virus lethal.

Objectives

The main objective of this thesis is the identification and characterization of the pathogenic determinants responsible for the lethal phenotype of a viral strain isolated from a fatal case. The putative virulence determinants observed in this viral strain (PB2 A221T; PA D529N and HA S127L) have not been previously associated with pathogenicity in A or B influenza viruses and their characterization may provide very valuable information to reveal which viral factors make an influenza virus lethal.

Objetives

1. To study the contribution of exclusive changes found in the F viral strain to their virulence *in vitro*

2. To evaluate the contribution of putative pathogenic factors of the F viral strain to the viral pathogenesis in the murine model for influenza virus infection.

3. To characterize virus spread potential to non-respiratory organs in *in vivo* infection with viruses carrying mutations found in the F strain

4. To investigate defective genome production of the F virus and viruses bearing exclusive changes found in F *in vitro* and to evaluate their contribution to viral pathogenicity *in vivo*.

I Materials

1. Cell lines

Madin-Darby canine kidney (***MDCK***) cells, 293T human embriotic kidney (***HEK293T***) cells, and human type II alveolar epithelial (***A549***) cells obtained from American Type Culture Collection (ATCC) were maintained in Dulbecco's minimal essential medium (DMEM; GIBCO) with 1% of non-essential amino acids (INVITROGEN) and 10% fetal bovine serum (FBS; LINUS) inactivated by incubation at 50˚C for 30 minutes. Propagation of cell lines was performed by serial passages every 3-4 days in order to avoid confluence. Cells were washed with 1x PBS (GIBCO) before every passage and then treated with 0.25% trypsin (SIGMA T4665) and 0.02% EDTA; in case of HEK293T cell line trypsin/EDTA mix was diluted 10 times. Cells were cultivated at 37˚C at atmosphere with 5% CO_2 and 98% humidity.

For experimental purposes, cell passages were done a day before the experiments and therefore recently grown cells were used for every *in vitro* study. Cell wash was performed with complete 1xPBS before any treatment (transfection, infection etc.).

2. Viral strains

Human viral isolates – H1N1 viruses used in this study were obtained from the Spanish National Influenza Centre during 2009 pandemic. Viruses were isolated from bronchealveolar lavages of one fatal influenza virus infected patient (**F**) and one patient who showed mild influenza symptoms (**M**) *(Rodriguez, Falcon et al. 2013)*.

Recombinant viruses –Recombinant influenza viruses bearing rare mutations detected in F strain and absent in M strain; HA S127L (CAL-HA), PB2 A221T (CAL-PB2), PA D529N mutations (CAL-PA), the combination of these polymerase changes (CAL-PB2/PA; F virus-like polymerase) or all possible combinations were generated on the A/California/04/09 (CAL) virus backbone.

Recombinant CAL viruses carrying mutations in M segment (M1 86S/M2 30N) *(Perez-Cidoncha, Killip et al.)* alone (CAL-M) or with mutated PA D529N (CAL-M-PA) were also generated.

All recombinant viruses were generated in the lab by Ana Falcón or Noelia Zamarreño (manuscript under review) following the protocol previously described *(Neumann, Watanabe et al. 1999, Falcon, Marion et al. 2004)*.

Virus ***ΔNS1***, corresponds to an influenza virus lacking NS1 protein *(Garcia-Sastre, Egorov et al. 1998)*. This virus is unable to counteract the IFN response.

3. Plasmids

PCAGGs plasmids are derived from CAL/04/09 strain (courtesy of Dr. Y.Kawaoka) and carried *wt* PB2, PA and NP proteins or PB2 segment with A221T change and PA segment with D529N found in the F virus.

PHH-NSCAT plasmid expresses the vRNA of cloramphenicol acetil transferase CAT in negative sense orientation under the Pol I promotor *(Gonzalez and Ortin 1999)*.

4. Bacteria strains

Ultracompetent XL10-Gold (STARTAGENE) with genotype Tet^r Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB $lacI^q$ZDM15 Tn10 (Tet^r) Amy Cam^r] were used for transformation.

5. Antibodies

Antibodies used for Western Blot analysis

PB1- anti viral PB1 polyclonal antibody obtained from immunized rabbit, used at 1:1000 dilution *(Gonzalez and Ortin 1999)*

NP- anti viral NP polyclonal antibody obtained from immunized rabbit, used at 1:5000 dilution *(Jorba, Coloma et al. 2009)*

ISG56 (IFIT1) – anti human ISG56 polyclonal antibody obtained from immunized goat used at 1:1000 dilution (IFIT1 (N-16), SantaCruz, sc-82946)

Mx 1/2/3 – anti human Mx1 polyclonal antibody obtained from immunized rabbit used at 1:1000 dilution, Mx 1/2/3 (H-285)(SantaCruz, sc-50509)

β-actin – anti human β-actin monoclonal antibody obtained from immunized mouse used at 1:2000 dilution (Sigma)

GAPDH – anti human polyclonal antibody obtained from immunized rabbit used at 1:1000 (ABCAM, ab-9485)

5.1 Antibodies used for flow cytometry analysis

Monoclonal antibodies used for flow cytometry were obtained from several commercial houses conjugated with different molecules as presented in Table 5.

6. Mice strains

The animal strain used in these studies was female BALB/c AnNHsd mice (6-7 weeks old) obtained from Harlan, USA and maintained in the animal facilities of National Centre of Biotechnology (CNB-CSIC).

7. Other material

Hu IFN-α2a - Recombinant human interferon alpha A (*PBL Assay Science, 11100-1*)

LB medium - **Luria Bertani Broth** (Sigma)

RPMI-1640 medium (*Invitrogen*)

Erythrocyte lysis buffer – 0.15M NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA (*Tritiplex*), pH=7.4.

Disruption buffer – 64% 10M urea, 21% SDS (20%), 15% β-mercaptoetanol, bromophenol blue

Transference buffer - 10% of 10X buffer (Tris 250mMl, glicin 1.92M, SDS 1%), 20% methanol (*Merck*).

Penicillin/Streptomycin – 100 IU/ml (*Gibco*, Thermofisher scientific, ref. 15140122)

II Methods

1. Viral manipulation

1.1 Viral stock generation

F, M and all recombinant viruses were collected in semi-confluent monolayer of MDCK cells when cytopathic effect was 75-100%. Primary obtained viruses (blind passage) were used in new infection at moi 10^{-3} for generation of secondary harvested viral stocks for further use. These 10^{-3} viral stocks were used in every experiment except for deep sequence analysis where stocks generated at moi 10^{-5} were analysed.

1.2 Viral titter estimation

Viral titters of both in vitro and in vivo samples were determined by standard plaque detection in MDCK cells. Briefly, approximately $6x10^5$ cells were infected with 100 µl of various serial dilution of viral samples in 5 µg/ml bovine serum albumin complete PBS (PBS-BSA) for 1h at 37˚C at atmosphere with 5% CO_2 and 98% humidity. Upon infection and removal of inoculum, cells were incubated for 72 hours in semisolid DMEM, 0.4% agar, 1 % DEAE-dextran y 2.5 µg/ ml trypsin medium at 37˚C at atmosphere with 5% CO_2 and 98% humidity. Viral titter was calculated by standard function by which number of plaques multiplied by factor of dilution of inoculum per ml.

1.3 Purification of viral particles

MDCK-infected cell culture supernatants were harvest at approximately 36-40 hours of infection and then centrifuged at 3110 g at 4°C for 10 min. Further on, supernatants were sedimented through a sucrose step gradient (TNE; 50% and 33% in 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 7.5) for 1 h at 27400 g at 4°C in a SW41 rotor. The 50 to 33% interphase was collected, diluted in TNE buffer, and pelleted through a cushion of 33% sucrose in TNE at 11200 g at 4°C in a SW28 rotor for 2 h. For purification of viruses isolated from infected mouse lungs, the previous protocol was used with modifications of the sucrose gradient volume and rotors according to sample.

2. Bacterial transformation

Ultracompetent XL10-Gold (STARTAGENE) *E.coli* bacteria (50µl) were incubated for 30 min on ice and then 10µl of purified plasmid was added. Mix was incubated for the next 20 min at 4˚C, then 1 min at 42˚C and finally 5 min at 4˚C when 1ml of LB was added. Cells were incubated for 1h at 37 ˚C. After centrifugation at 8000rmp for 3 min at RT, 200µl of supernatant was left with cell pellet, mixed gently and plated on the AmpLB agar plate (ampicillin 0.1mg/ml). Plates were incubated O/N at 37 ˚C. Bacteria colonies observed the next day were collected and grown in 5ml liquid AmpLB at 37 ˚C O/N.

3. DNA and RNA manipulation

3.1 Mutagenesis of pCAGGs-PB2 and PA plasmids derived from CAL/04/09

Specific mutations were engineered in expression pCAGGS plasmids derived from the CAL strain using the QuickChangeTM site-directed mutagenesis kit (Stratagene) as recommended by the manufacturer. These materials were developed using the Licensed technology (Kawaoka-P99264US Recombinant Influenza viruses for vaccines and gene therapy). Primers used for site-directed mutagenesis of pCAGGS plasmids can be found in the table below *(Table 1)*.

Plasmids	Primer sequence
pCAGGs–mutPB2	[Phos]GGTTTCTCCCAGTAACCGGCGGAACAGGCAGTG
pCAGGs-mutPA	[Phos]GTATGGAGTTCTCACTCACTAACCCGAGACTGGAGCCACAC

Table 1. Sequence of specific site-directed mutagenesis primers for pCAGGS plasmids

pCAGGs–*mut*PB2 and pCAGGs-*mut*PA were generated by PCR preheating the total mix 1 min at 95˚C and followed by 35 rounds for 1min at 95˚C, 1min at 55˚C and 7 min of elongation at 65˚C. Amplified products were purified adding 1%TNE, 36% of miliQ H_20 and 50% of phenol, mixed and centrifuged at 12000rmp on 4˚C for 5 min. Pellet was washed with 2.5 volumes of 100% ethanol and 2µg of glycogen left for o/n precipitation at - 20˚C. Precipitate was then centrifuged at 12000rmp at 4˚C for 30 min and pellet washed twice with 70% ethanol and once with 100% ethanol, with short centrifugation of 5 min at 12000rmp at 4˚C between each wash. Dried pellet was re-suspended in miliQ H2O.

After mutagenesis, the plasmids were transformed in bacteria as indicated above (Methods 2). Selected bacteria colonies were grown and plasmids were purified using *QIAprep Spin Miniprep Kit* (*Quiagen*) following the protocol recommended by manufacturer. To confirm the incorporation of point mutations, pCAGG-PB2 and PA plasmids were sequenced as recommended by *Macrogen*, using both forward and reverse primers *(Table 2)*.

Gene	Primer sequence
v PB2	Fw: 5'-AGGAATGGCCCAACAACAAGT-3' Rev:5'-TCCTCAACCCTATTGCTGCC-3'
v PA	Fw:5'-AGAAGATGTTGCCCCGATTGA-3' Rev:5'-TCCAATAGAGCCTTCCTCCA-3'

Table 2. Oligomers used for sequence analysis of introduced mutation PB2A221T and PAD529N in pCAGG plasmids

3.2 RNA extraction

3.2.1 Viral RNA

For viral RNA isolation, viral stocks and purified virions were treated with 0.5% SDS and 200µg/ml proteinase K in TNE for 2h at 37ºC *(Rodriguez, Falcon et al. 2013)* followed by extraction with phenol-chloroform-isoamylalcohol-hydroxyquinolein and ethanol precipitation. DNA was removed by treatment with DNAse I recombinant, RNase-free (Roche), following instructions of the manufacturer.

3.2.2 Cellular RNA

RNA isolation from cell cultures and animal tissues was performed with TRIzol (Invitrogen, 15596018)/chloroform (MERCK)) extraction according to manufacturer´s instructions. Samples collected with TRIzol reagent and 1/5 volume of chloroform were incubated at RT for 5 minutes and then centrifuged at 13000rmp at 4˚C for 20 min. Aqueous phase was collected and 1 volume of isopropanol was added. Samples were precipitated O/N at -20˚C. Precipitate was then centrifuged at 12000rmp for 30 min at 4ºC and pellet washed twice with 70% ethanol and once with 100% ethanol, with short centrifugation of 5 min at 12000rmp at 4˚C between each wash. Air dried pellet was re-suspended in RNase free H_2O. DNA was removed by treatment with DNAse I recombinant, RNase-free (Roche), following instructions of the manufacturer.

Quantity and quality analysis of RNA samples were performed by absorbance measuring at 260nm by *NanoDrop ND*-1000. All RNA samples were stored at -80˚C.

3.3 PCR

3.3.1 Quantification of gene expression (qPCR)

RNA samples obtained from various in vitro and in vivo samples were extracted as previously described (Methods 3.2). Reverse transcription of RNA samples was performed by *High Capacity RNA Transcriptase Kit* (*Applied System, Thermo Fisher Scientific*) following the manufacture recommendations, using random primers, provided within *High Capacity RNA Transcriptase Kit* (*Applied System, Thermo Fisher Scientific*) except for transcription of NEP viral gene where PolydT_{16} of *TaqMan Reverse Transcriptase Reagans* (*Applied System, Thermo Fisher Scientific*) were used as a primer. RNA samples used in these RT-PCR reactions were 100ng. PCR was performed in Applied Biosystems 2720 Thermal Cycler.

For estimation of gene expression we used quantitative PCR approach. Previously isolated and transcribed RNA samples were quantify with this technique by *Power SYBR green PCR master mix* (*Applied Biosystems, 4369679*) following the manufacturer recommendations, using 10% of reverse transcribed cDNA, and 5% of 10mM primers from the table below *(Table 3)*. For estimation of gene expression we used Thermocycler 7500 Real Time PCR Biosystems 2 min at 50°, 10 min at 94° 40 cycling stages of 15s at 94° and 1min at 60°. As a standard curve plasmids with integrated fragments of ISG56 and MxA genes quantified by absorbance measured at 260nm by *NanoDrop ND*-1000 were used. In case of NEP mRNA detection, fragments were previously amplified from RNA sample of A549 infected cells then

quantified and used as standard curve. Human 28SrRNA and mouse β-actin were used as internal control in each sample; therefore they were not quantified but were used for normalization of data obtained.

3.3.2 Detection of defective genome (DGs)

Genes	Primer Sequences
v NEP	Fw: 5'-GTCAAGCTTTCAGGACATACTTATG-3' Rev: 5'-TGCTCTGGAGGTAGTGAAGGT-3'
h ISG56	Fw : 5'-GGGCAGACTGGCAGAAG-3' Rev: 5'-CTATAGCGGAAGGGATTTGA-3'
h MxA	Fw: 5'-ACAGGACCATCGGAATCTTG-3' Rev:5'-CCCTTCTTCAGGTGGAACAC-3'
h 28SrRNA	Fw :5'-GAAGGTGAAGGTCGGAGTC -3' Rev: 5'-GAAGATGGTGATGGGATTTC-3'
m β-actin	Fw: 5'-GGCTGTATTCCCCTCCATCG-3' Rev: 5'-CCAGTTGGTAACAATGCCATGT-3'

Table 3. Primer sequences used for quantification of gene expression in infected cells and viral mRNA transcription

For detection of DGs, 100 ng RNA samples extracted from purified viral particles (infected cells, infected mice lungs) or infected mice lungs were used for amplification using one step *Titan One Tube RT-PCR Kit* (Roche) applying all the manufacturer recommendations. RT-PCR for the PA and PB2 segments were used to determine the presence of DGs originated from these two segments. To detect full-length (PA and PB2) segments, internal primers were used to amplify central fragments. To detect DGs, same RNA sample and external primers of the PA and PB2 segments were used in a separate reaction. Short amplification time was applied for both external and internal primers (not provided due to intellectual properties issues). Model of detection of both full length and defective gene segments is shown in the Figure 9.

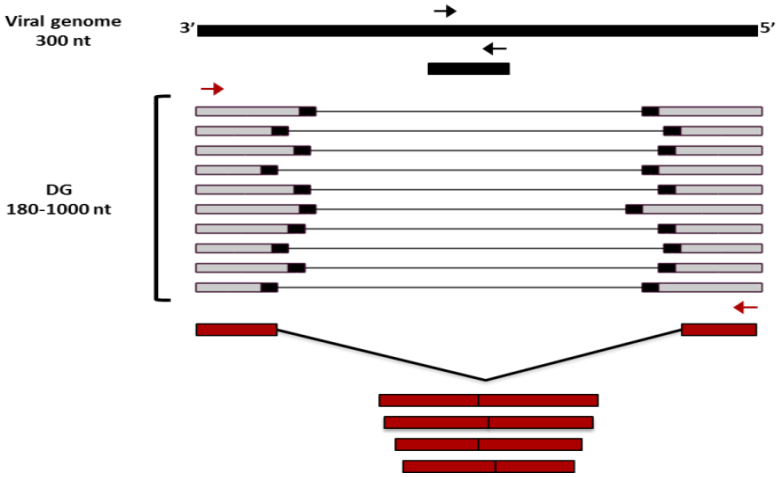


Figure 9. Model of detection of defective and full length gene segments during in vivo and in vitro infection. Central fragment *(black)* of the whole viral gene is amplified by specific internal primers *(black arrows)*, defective genomes *(dark red)* with central deletion were amplified by a specific 3' and 5'-end gene specific primers *(dark red arrows)* in two different RT-PCR reaction.

The reverse transcription reaction was performed for 30 min at 42°C and then PCR was performed for 35 rounds of 94°C for 30 s, 59°C 30 s, and 68°C for 40 s for PB2 gene amplification, and 35 rounds of 94°C for 30 s, 53°C 30 s, and 68°C for 40 s for PA gene amplification at Applied Biosystems 2720 Thermal Cycler. As a specificity control, the primers and RT-PCR conditions for DGs amplification were used with a plasmid encoding the full-length PA segment, and no amplification product was obtained.

3.4 Deep sequencing

Recombinant viruses were sequenced with TruSeq v3 chemistry and 50 bp single reads on an Illumina HiSeq 2000.

3.4.1 Deep sequencing data analysis

3.4.1.1 Short read alignment against the influenza genome

For each sample, FASTQ sequences were aligned against the influenza (A/California/04/09) genome with TopHat2 (Kim, Pertea et al. 2013), allowing intron size ranges between 5 and 10^5 nucleotides. Samtools (Li, Handsaker et al. 2009) was used to extract reads that aligned as two separate fragments (jumping reads) from the BAM files generated by TopHat2. Only reads split into two fragments that align at distant segment positions (jumping reads) and with both fragments of four or more nucleotides were selected.

3.4.1.2 Viral sequence consensus determination

To determine the consensus sequence of each virus, coverage and nucleotide composition of aligned reads were analysed. Nucleotide positions with an identity of ≥75% were considered. Samtools mpileup (Li, Handsaker et al. 2009) and in-house php scripts were used.

3.4.1.3 Junction quantification, filtering and normalization

Jumping reeds that aligned at least 4 out of 50 nucleotides, followed by ≥100 nucleotides gap and then aligned the rest of the sequence were considered defective genome junctions. Only defective segments generated by two or more jumping reads spanning exactly the same coordinates and that generate a theoretical final RNA segment size of ≤1000 nucleotides were selected. For samples from the year 2009 (F and M virus), read orientation could not be determined as the sequencing protocol applied was not strand-specific. In the remaining samples, only reads from genomic RNA strand were counted. For each sample, values for jumping reads per million (RPM) were calculated as the sum of all selected jumping reads divided by the number of total reads that aligned with the influenza genome, then multiplied by 10^6. For analysis of jumping reads per segment, RPM values were normalized to total reads aligned with each RNA segment of the genome.

4. Protein accumulation analysis

4.1. Enzyme-Linked ImmunoSorbent Assay (ELISA)

For chloramphenicol acetyl transferase detection CAT ELISA (Roche, 11363727001) commercial kit was used following the manufacture recommendations. Accumulation of CAT enzyme was quantified by measuring absorbance at 405nm wavelength on microplate reader (EZ Read 400, Biochrom).

4.2. Western blot

Samples were collected in disruption buffer (Materials 7), sonicated (S3000-010 Misonix Incorporated, New York, USA) 3 cycles for 10 s in water. After protein denaturation at 100˚C for 10 min, samples were centrifugated at 12000rmp at 4˚C for 5 min. Proteins were separated by 8% SDS-PAGE gel electrophoresis (40 mA, 150V) and transferred to polivinil floured filters (PVDF membrane, *Immobilon P Milipore*) in transference buffer (Materials 7) O/N at 200mA at 4˚C in semi-liquid electrotransfering system (*Biorad*).

For western blotting of viral proteins the membranes were saturated with 3% bovine serum albumin for 2 h and then incubated with the primary antibodies (diluted in PBS-0.05% Tween 20) for 1 h at room temperature. For Western blotting of human proteins, the membranes were saturated with TBS-5% milk for 2h at room temperature or o/n at 4ºC and then incubated with the primary antibodies (diluted in TBS-0.05% Tween 20) overnight at 4°C. The filters were washed with PBS or TBS containing 0.25% Tween 20 and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. After further washing, the filters were developed by enhanced chemiluminescence by membrane incubation with solution (100mM Tris pH8.5, 250mM luminol, 90mM cumaric acid, 1µl H_2O_2) for 1 min. Exposition of X-ray films sensible for chemiluminiscence to a PVDF membrane was between 1 s-10 min, depending on experiment. Visualisation of the signal on the X-ray films with detected protein was performed by M-35 X-OMAT Processor (*Kodak*). List of antibodies used for Western Blot and conditions were described in the table below *(Table 4)*.

Antibody	Origen	Comertial house	Reference	Delution
Polyclonal anti viral PB1	Rabbit	Juan Ortíns lab, CNB	Gonzalez and Ortín 1999	1:1000
Polyclonal anti viral NP	Rabbit	Juan Ortíns lab, CNB	Jorba, Coloma et al. 2009	1:5000
Polyclonal anti h ISG56	Goat	SantaCruz	sc-82946	1:1000
Polyclonal anti h Mx1/2/3	Rabbit	SantaCruz	sc-50509	1:1000
Polyclonal anti h GAPDH	Rabbit	Abcam	ab-9485	1:5000
Monoclonal anti h β-actin	Mouse	Sigma	A5441	1:2000
Anti rabbit IgG peroxidase	Goat	Sigma	A9169	1:20000
Anti goat IgG peroxidase	Rabbit	Sigma	A5420	1:20000
Anti mouse IgG peroxidase	Goat	Biorad	170-6516	1:20000

Table 4. Antibodies used for Western blot detection of proteins during viral infection

5. *In vivo* studies

5.1 Ethics statement

All the procedures that required the use of animals complied with Spanish and European legislation concerning vivisection and the use of genetically modified organisms, and the protocols were approved by the National Centre for Biotechnology Ethics Committees on Animal Experimentation and the Consejo Superior de Investigaciones Científicas (CSIC) Bioethics Subcommittee. In particular, we follow the Guidelines included in the current Spanish legislation on protection for animals used in research and other scientific aims: RD 1201/2005, 10 October and the current European Union Directive 86/609/CEE, DOCE 12.12.86 (N.L358/1 to N.L358/28) on protection for animals used in experimentation and other scientific aims.

5.2 In vivo infections

All in vivo infections were performed on animal under anaesthesia, induced by exposition to vapours of *Isofluorane (ISOflo -Ecuphar*) for a few minutes.

To calculate lethal dose 50 (LD_{50}), 4 female BALB/c AnNHsd mice (6-7 weeks old) were infected intranasally with several doses (10^2-10^6 pfu/50µl DMEM) of each of the recombinant influenza viruses described here, or were mock-infected (50µl DMEM). The animals were monitored daily for clinical signs (piloerection, mobility and wellbeing) and body weights for two weeks. For ethical reasons, mice were euthanized when they presented 25% body weight loss.

For the kinetics experiment, 5 female BALB/c mice (6-7 weeks old) were infected intranasally with a sublethal dose (10^3 pfu/50µl DMEM) of recombinant wild-type CAL, CAL-HA, CAL-PA, CAL-PB2 or CAL-PB2/PA influenza viruses, or were mock-infected (50µl DMEM). At 1, 2, 4 and 7 dpi, mice were euthanized by CO_2 inhalation and necropsied.

5.3 Organs extraction

Left kidney, heart and lungs were surgically extracted from all sacrificed animals infected with sublethal viral doses at different time points of infection (1^{st}, 2^{nd}, 4^{th} and 7^{th} dpi). Heart and kidney samples were extracted as the whole organ and kept at -80˚C. Lung sample of each mice were divided in to 4 samples: superior and post-caval lobes were used for RNA analysis, kept at -80˚C, middle and inferior lobes for viral titer estimation, kept at -80˚C, while left lobe was used for histology analysis (lower left lobe) and flow cytometry analysis (upper left lobe).

5.4 Viral titer estimation in extracted organs

Tissue samples were homogenized (2x vol/weight) in PBS-0.3%-BSA-penicillin/streptomycin (100 IU/ml) using an Electronic Douncer (IKA T10 basic, Workcenter). Lung and heart samples were homogenized 1min and kidney samples 40 s at max speed at 4˚C. Debris was pelleted by centrifugation (1500rmp, 5 min, 4˚C). Viral titter was determined by standard plaque assay on MDCK cells as described before (Methods 1.2).

5.5. Preparation of lung cell suspension

Upper left lungs were kept in RMPI medium at 4˚C. Tissue samples were grinded into very small pieces prior to digestion with 180 µg/ml *liberase* (*Roche*) and 40 µg/ml *DNase I* (*Roche*) in RMPI medium for 30 minutes at 37˚C. Digested fragments were filtrated with *40mm Nylon Cell Strainer* (*BD Falcon*) and resuspended with RMPI-3%FBS. After the centrifugation of samples (1640 rmp, 5 min, 4˚C) additional step for erythrocyte lysis were performed. Cell pellet was incubated for 1.5-2 min with 1ml of erythrocyte lysis buffer (Material 6) at RT. Lysis is inhibited by adding 9ml of PBS-5mM EDTA-3%FBS. Samples were then filtrated again with 40mm Nylon Cell Strainer and pelleted by centrifugation (1640rmp, 5 min, 4˚C).

5.6 Flow cytometry analysis

Cell suspensions were distributed in 96 wells plate and first incubated with violet LIVE/DEAD due (*Invitrogen*) for 30 min at 4˚C, washed 2 twice with PBS and then incubated for 15 min at 4˚C with Fc block CD16 rat antibody. Samples were analysed by staining cell suspension with conjugated antibodies *(Table 5)* with 30 µl antibody mix in 1XPBS for 30 minutes at 4˚C in the dark. Samples were than fixed by incubation with 4% formaldehyde for 20 min, pelleted by centrifugation (700 rmp, 5 min, 4˚C) and washed once with PBS. After centrifugation (700 rmp, 5 min, 4˚C), cells were resuspended in 0.4ml PBS and kept at 4˚C O/N in the dark. Flow cytometric analysis was performed on a cytometer LSR II (BD Biosciences).Data were analysed using *CellQuestPro* software.

Receptor	Conjugate molecul	Clon	Isotype origin	Commercial house	Delution
CD16	-	93	IgG2bk/rat	BACKMAN	1:100
CD45	Percp-Cy5.5	30-F11	IgGb/ rat	BioLEGEND	1:200
CD11b	PeCy7	M1/70	IgG2bk/ rat	BioLEGEND	1:100
CD11c	APC	N418	IgG/ hamster	eBIOSCIENCE	1:100
CD8	APCeF780	53.67	IgG2ak/ rat	eBIOSCIENCE	1:200
Ly6G	PE	1 A8	IgG2ak/ rat	BDBIOSCIENCE	1:100

Table 5. Antibodies used for cell suspension staining

1. Study of the contribution of changes found in the F viral strain to its pathogenicity *in vitro* and *in vivo*

To characterize the putative F virus virulence determinants, we generated recombinant influenza viruses bearing HA S127L, PB2 A221T (CAL-PB2) or PA D529N mutations (CAL-PA) or the combination of these changes (CAL-PB2/PA; F virus-like polymerase) in the A/California/04/09 (CAL) as backbone. CAL virus is one of the first swine-origin H1N1 influenza virus isolated from a 10-year old patient in the USA. Viral particles of this virus are more filamentous than other H1N1 strains. CAL virus is shown to be more pathogenic in infected mammals (mice, ferrets and non-human primates) than contemporary H1N1 strains *(Itoh, Shinya et al. 2009)*. It is oseltamivir and zanamivir sensitive but, as all pandemic strains are, amantadine resistant *(Swiss Institute of Bioinformatics 2009)*. To test if any of detected changes was responsible for the behaviour of the F strain, the growth kinetics of these viruses were analysed in human alveolar A549 cells (Methods, 1.2). Statistical analysis were performed by unpaired, two tailed Student t-test.* p<0, 05.

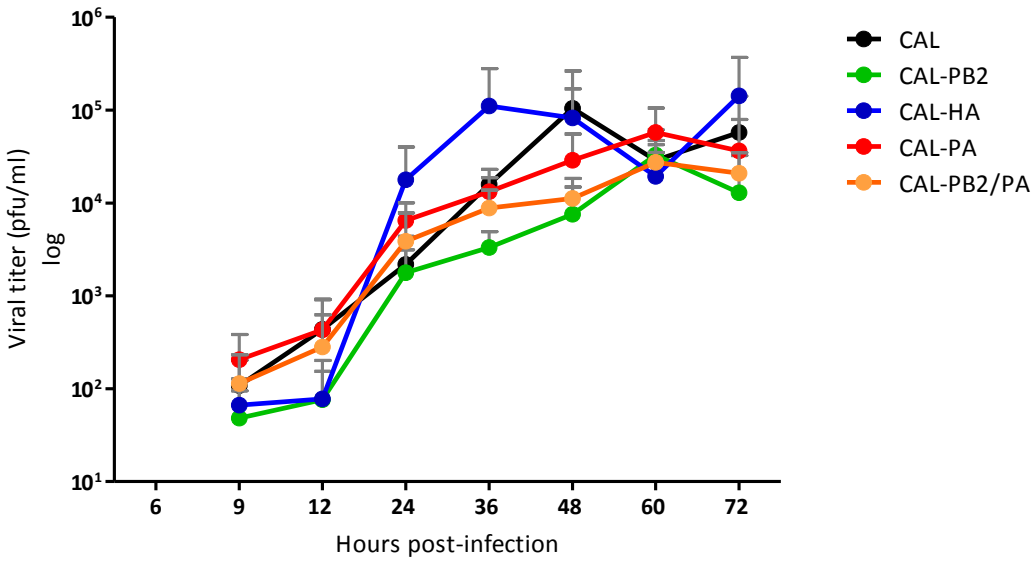


Figure 10. Growth kinetics of recombinant viruses in human alveolar cells. A549 cell line was infected at moi 10^{-3} pfu/cell for 72 hours. Samples were obtained at different time points for estimation of viral titer. Viral yield was determined by plaque assay in the MDCK cells at different time points.

All recombinant viruses, including CAL-PB2/PA (F-like-polymerase virus) showed similar growth rate and viral titers at 72 hours of infection *(Figure 10)*. The CAL-PB2 recombinant virus had a lower growth rate than others, which is statistically significant only at 36 hours of infection, when compared to both CAL and CAL-PA recombinants. All recombinants bearing mutation in HA segment, single or in combination with others, showed a tendency of reaching higher viral titers from 24 to 48 hours upon the infection, although this growth rate was not statistically significant (data not shown).

Increased polymerase activity constitutes an important virulence factor in influenza virus. In order to examine if any of these mutations in PA and/or PB2 polymerase subunits modify the viral polymerase activity, we performed *in vivo*

reconstitution of viral ribonucleoproteins (vRNPs). Cells were transfected with plasmids expressing the wild-type or mutant polymerase subunits and NP together with a plasmid that expresses a flu-like chloramphenicol acetyltransferase (CAT) in negative sense orientation. The CAT enzyme can only be transcribed if viral polymerase complex if fully functional in transfected cells. The viral polymerase activity is determined as the relative CAT accumulation which is measured by CAT enzyme activity (Methods 4.1).

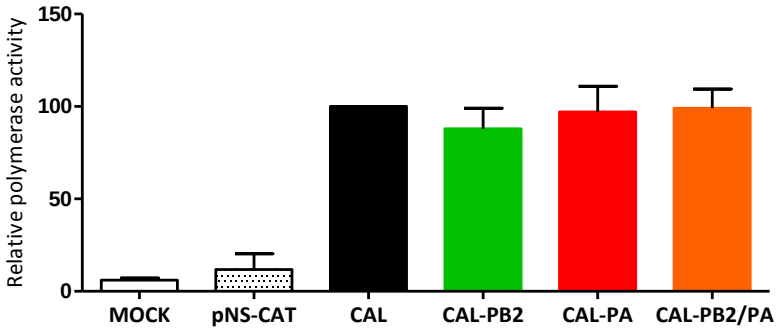


Figure 11. Relative polymerase activity of wild-type or mutant reconstituted RNPs. HEK293T cells were transfected with plasmids expressing CAT in antisense orientation, NP, PB1, and *wt*PB2 and *wt*PA (CAL), or the corresponding subunits expressing mutated PB2 A221T (CAL-PB2), mutated PA D529N (CAL-PA) or mutated PB2 A221T and PA D529N (CAL-PB2/PA). At 24 h post-reconstitution, CAT protein in total cell extracts was analysed by ELISA. In MOCK, plasmid expressing PB1 was omitted. pNS-CAT indicates CAT accumulation in cells transfected exclusively with pHHNS-CAT plasmid. Three independent experiments were performed; values shown as means (%) ± SD. Statistical analysis done by unpaired, two tailed, Student t-test showed no significant differences in polymerase activity of these recombinants.

Similar CAT activity was observed in all reconstituted RNPs, suggesting that neither single mutations PB2 A221T or PA D529N nor both of them together, affect the activity of the polymerase complex in transfected cells *(Figure 11)*. Both results indicate that none of the evaluated mutations or combinations of them *(Figure 10)* incorporated in Cal/04/2009 H1N1 pandemic strain backbone are responsible for F strain virulent phenotype in cell culture.

2. Characterization of recombinant viruses containing changes present in the F isolate in mouse infection model

2.1 Determination of LD_{50}

It has been previously described that in animal model, theF virus was more pathogenic than the M, causing significant weight loss and 50% survival drop by the 6^{th} day of infection *(Rodriguez, Falcon et al. 2013)*. Since there was no correlation between the virulence of particular recombinant virus and the F strain in infections in cell culture, we performed *in vivo* experiments to evaluate the effect of mutations in more complex infection system. To evaluate the pathogenicity of the recombinant viruses, mice were intranasally infected with various doses of the different recombinant viruses. Body weight loss, as a symptom of illness *(Figure 12)*, and survival rate *(Figure 13)* were monitored daily for two weeks. Viral dose that causes 50% of mortality (LD_{50}) was calculated for each recombinant virus (Methods 5.2.)

VIRUS	LD_{50}
CAL	1.6×10^5
CAL-PB2	$>10^6$
CAL-PA	3.1×10^3
CAL-PB2/PA	3.5×10^4
CAL-HA	$>10^6$
CAL-HA/PB2	$>10^6$
CAL-HA/PA	$>10^6$

Table 6. Evaluation of pathogenicity of recombinant viruses in mice. Balb/C female mice (n = 4) were inoculated intranasally with 10^6–10^2 pfu of each recombinant virus. The dose that causes 50% mortality of mice in each infection (LD_{50}) is shown.

Using high viral doses of infection, we observed that mice infected with recombinant viruses that carried HA change and PB2 change showed signs of mild illness which induced lower weight loss when compared to CAL recombinant *(Figure 12)*. These recombinants (CAL-HA and CAL-PB2) showed completely different pathogenicity pattern in murine model than viruses with PA change in its genome.

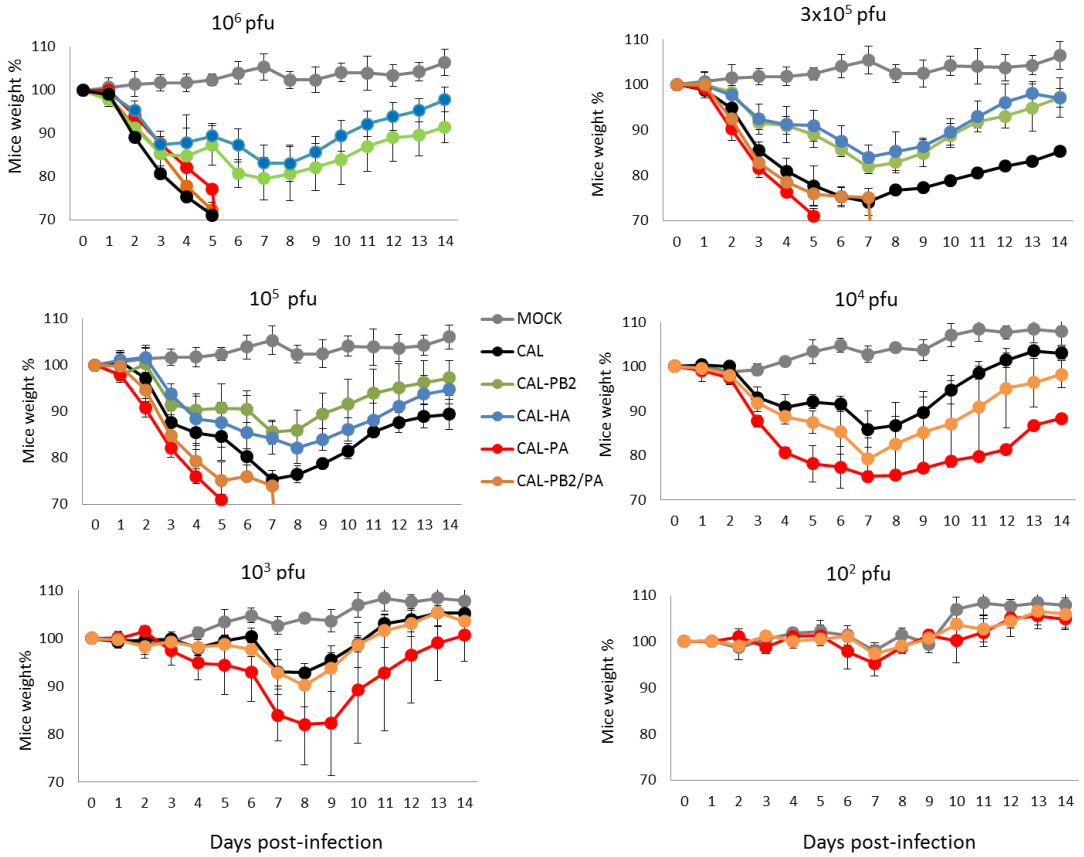


Figure 12. **Pathogenicity of recombinant viruses in *in vivo* infections.** Mice (*n* = 4) were intranasally infected with different viral doses and monitored for weight loss as a sign of infection during 14 days. Due to ethical reasons animals were euthanized when they lost 25% of their original body weight.

CAL, CAL-HA, CAL-PB2, CAL-PA and CAL-PB2/PA viruses showed LD_{50} of 1.6 x 10^5, >10^6, >10^6, 3 x 10^3 and 3.5 x 10^4, respectively *(Table 6)*. These data confirmed that CAL virus is pathogenic in mice, as it has been previously shown *(Itoh, Shinya et al. 2009)*, and indicated that the PA D529N mutation greatly increases its' pathogenicity, suggesting a decisive effect of this polymerase change on disease outcome. CAL-PB2 and CAL-HA were greatly attenuated compared with the CAL strain. Furthermore, all recombinants that carried HA change alone or in combination with others changes in PA and PB2 gene segments, showed decreased pathogenicity in murine model *(Table 6)*. These data indicate that mutation detected in HA segment of F viral strain is not responsible for its pathogenicity in mice. Therefore, recombinant viruses with HA mutation have been excluded from further *in vivo* studies. On the other hand, reconstitution of the F-like polymerase-containing virus (CAL-PB2/PA) led to lower pathogenicity compared with CAL-PA virus.

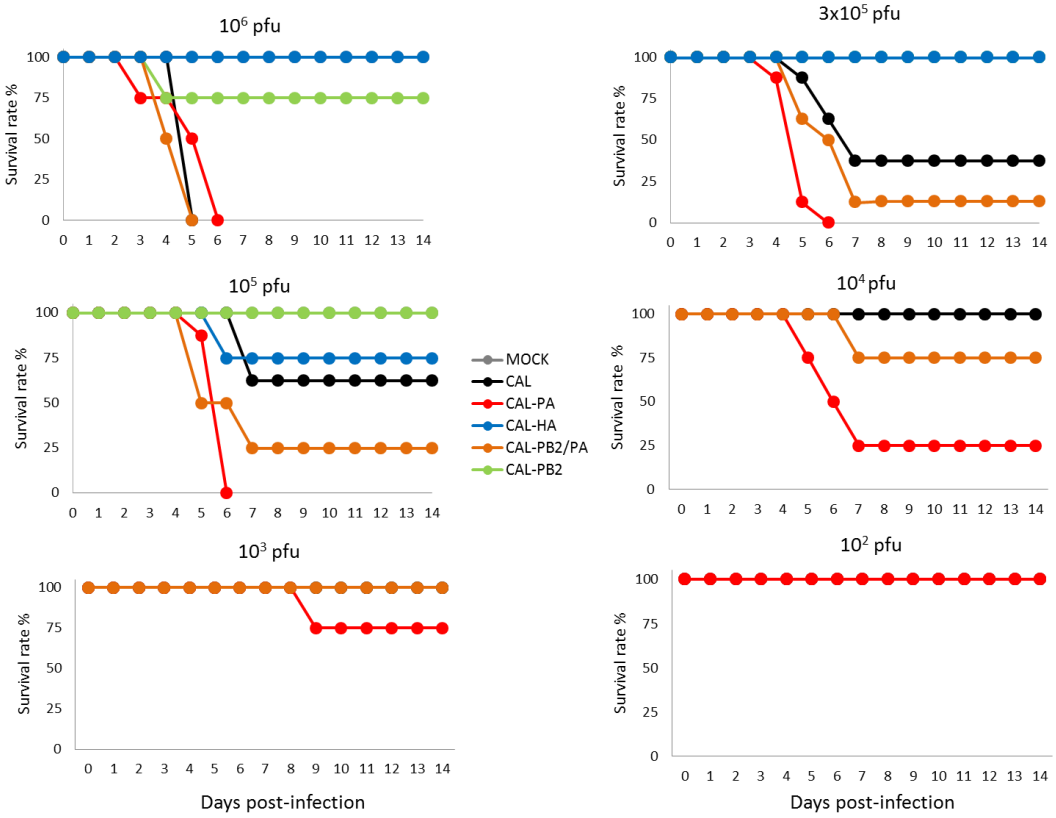


Figure 13. Pathogenicity of recombinant viruses in in vivo infections. During 14 days of infection, survival rate was monitored on mice (*n* = 4) intranasally infected with different viral doses. Due to ethical reasons animals were euthanized when they lost 25% of their original body weight.

These data show that recombinants with the PA mutation present in the F strain are more pathogenic in mice experimental model. PA mutation alone or together with PB2 mutation, retains the lethal phenotype showed by F strain in mice model and recombinants with PA or both PA and PB2 mutations cause more mortality than control CAL virus. Summarizing, introduction of PA D529N mutation in Cal/04/09 strain increases its pathogenicity *in* vivo. Moreover, a viral dose that drops survival rate 50% in mice infected with the F strain is lethal in animals infected with CAL-PA virus. CAL-PA recombinant virus is even more pathogenic in the mice model than F strain.

Pathogenicity of influenza virus depends on various and complex features. Final outcome of infection involves several factors such as the nature of the virus and the host and their interplay. To further evaluate the differences in the pathogenicity of these viruses, mice were infected with a sub lethal dose of the recombinant CAL, or the different recombinant viruses, or were mock-infected (Methods 5.2). Tissue samples were collected on the 1st, 2nd, 4th and 7th day post-infection (dpi) to determinate viral titers, viral spread, genome expression and immune response (Methods 5.3- 5.5). Each lung was divided into 4 samples: superior and post-caval lobes were used for determination of viral titers, inferior and middle lobes for RNA extraction while left lung was divided into 2 samples. Upper left lung lobe was used for flow cytometry analysis of innate immune response and lower left lobe for histological analysis *(Figure 14)*.

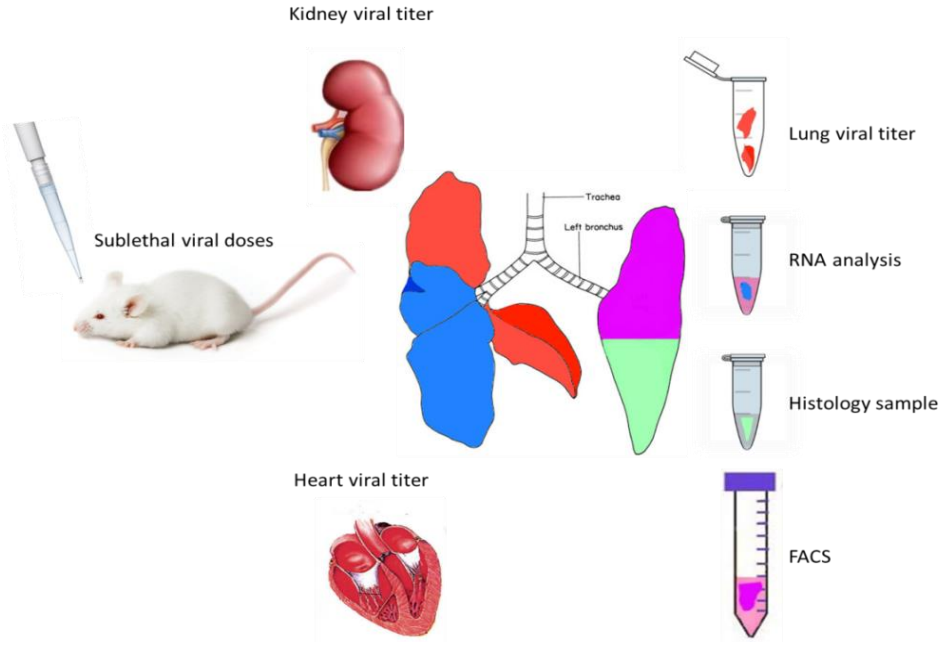


Figure 14. In vivo analysis of recombinant virus infection. Balb/c Mice (*n* = 5) were infected with a sublethal viral dose (10^3 pfu) of each recombinant virus. Organs were collected at different time points of infection in order to analyse pathogenicity and viral spread capacity of each recombinant virus.

The detection of H1N1 influenza virus in organs different from the lung, such as kidney has been described *(Watanabe 2013)*, as well as heart dysfunction for severe influenza H1N1 infected patients *(Komai, Nakazawa et al. 2011, Jeyanathan, Overgaard et al. 2013)*. Additionally we have previously described the presence of infectious particles in higher proportion and remaining at later dpi in the hearts of F virus compared to M virus infected mice *(Rodriguez, Falcon et al. 2013).* Therefore, we tested the possible presence of the different influenza recombinant viruses in these organs, in addition to lungs.

2.2 Replication of recombinant viruses in lungs of infected mice

Viral replication in lungs was evaluated at several points during the infection. Lungs lobes weight were measured and diluted in PBS-BSA (Methods 5.4). Data are presented as the pfu per grams of tissue. The weight of superior and post-caval lobes used for determination of viral titer was measured to examine possible liquid accumulation or other tissue damage caused by an inflammation process in infected lungs. Results presented in Figure 15, show lower lung weights of mice infected with CAL-PA virus but with differences not statistically significant.

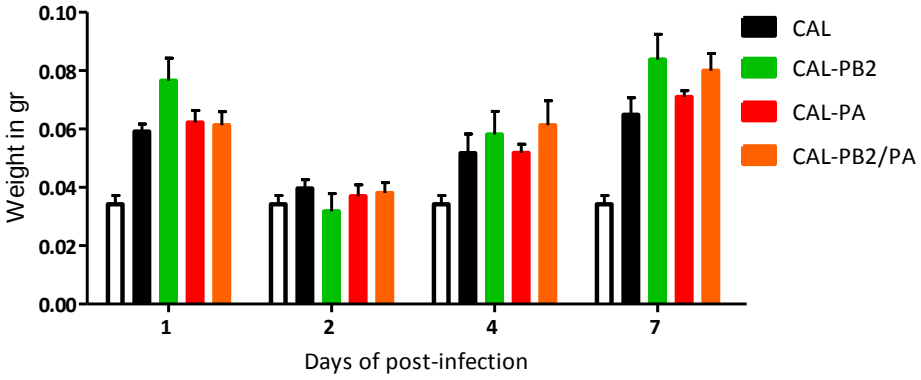


Figure 15. Weight of superior and post-caval lung lobes. Samples were used to examine possible liquid accumulation or other tissue damage caused by an inflammation process in infected lungs.

Data show the highest viral titers in mice infected with CAL-PA recombinant virus *(Figure 16)*.

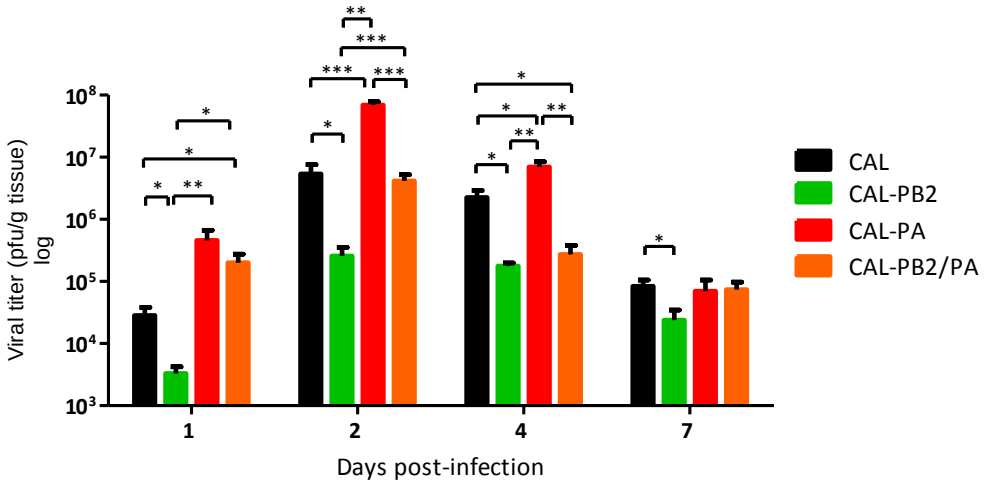


Figure 16. Viral titer in the lungs of infected mice. Mice (*n* = 5) were inoculated intranasally with a sub lethal dose (10^3 pfu) of each recombinant virus or PBS (MOCK) as control. At indicated dpi, viral titers were determined in the lungs (pfu/g tissue) by plaque detection in MDCK cells, statistical analysis was performed by unpaired, two tailed, Student t-test *** p<0.0001, ** p<0.005, *p<0.05

Interestingly, viral titer of CAL-PA recombinant is not significantly higher than CAL and CAL-PB2/PA viruses at early infection (1 dpi). Recombinant virus CAL-PB2 produced low viral titers throughout all the infection, which were significant, compared with the recombinant Cal/04/09 virus.

2.3 Replication of recombinant viruses in extra-pulmonary organs of infected mice

Influenza A virus can spread to extra-pulmonary organs, which may contribute to its pathogenicity. Previous study showed that F virus was capable of spreading outside lung tissue in animal model *(Rodriguez, Falcon et al. 2013, Watanabe 2013)*.

Analysing our data *(Figure 12 and 13)* we observed a high mortality in animals infected with high doses of CAL-PA recombinant, that occurred in mice which did not get below 77% of original weight and did not reach the limited 25% weight lost *(Table 7)*. In fact, animals infected with CAL and CAL-PB2/PA recombinants with the same high viral doses, lost weight faster than animals infected with CAL-PA recombinant, and only one CAL-PB2/PA infected mouse suffered sudden dead. This might indicate that besides usual symptoms, infection with recombinant viruses containing PA mutation causes atypical health conditions and sudden death in infected animals.

Virus	0 dpi	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi
MOCK	100,0	101	101	102	102	102	104,0
CAL	100,0	99	92	85	78	72	
CAL-PB2	100,0	98	91	85	85	87	80
CAL-PA	100	99	92	86	83	**77**	
	100	100	97	88	81	**78**	
	100	101	**95**				
	100	99	94	88	**82**		
CAL-PB2/PA	100	98	88	79	74		
	100	97	90	82	**77**		
	100	100	89	81	76	71	
	100	100	90	80	75		

Table 7. Animals infected with CAL-PA recombinant virus experiencing sudden death early in infection. Mice (*n*=4) were intranasally infected with 10^6 pfu viral dose. Weight loss of animals infected with CAL, CAL-PA and CAL-PB2/PA recombinant viruses at several days post-infection is expressed taking as 100% the initial weight before infection. The boxed data belong to mice presenting sudden death.

It has been reported that influenza infection can induce serious heart dysfunctions in patients as well as acute viremia. Thus, we monitored possible viral spread into extra-pulmonary tissues with focus on highly vascularized organs such as kidney and heart. First, we evaluated infection of kidneys *(Figure 17)* by the different recombinant viruses. Viral titer was determined by plaque detection on MDCK cells and viral particles were not present in kidney samples of any infected animal.

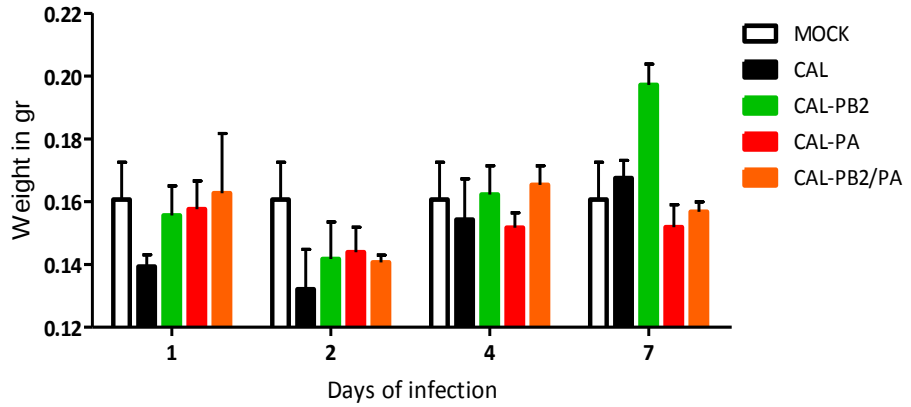


Figure 17. Kidneys weight of the mice infected with the different recombinant viruses. Kidneys of mock- and influenza-infected mice at different time points of infection were excised and weighed; Statistical analysis was performed with unpaired, two tailed Student t-test.

Seasonal infection may cause severe illness and death in patients with previously diagnosed heart conditions. Even in the healthy individuals influenza infections may lead to serious cardiovascular dysfunctions that may include myocarditis, acute myocardial infarction and hypertrophic cardiomyopathy *(Mamas, Fraser et al. 2008, Komai, Nakazawa et al. 2011)*. Additionally, it has been previously shown in our laboratory the presence of viral particles in the heart of mice infected with the F strain on the 2nd and 4th day of infection *(Rodriguez, Falcon et al. 2013)*. Thus, both, heart weights and viral titer in hearts were examined at different stages of infection with the different recombinant viruses.

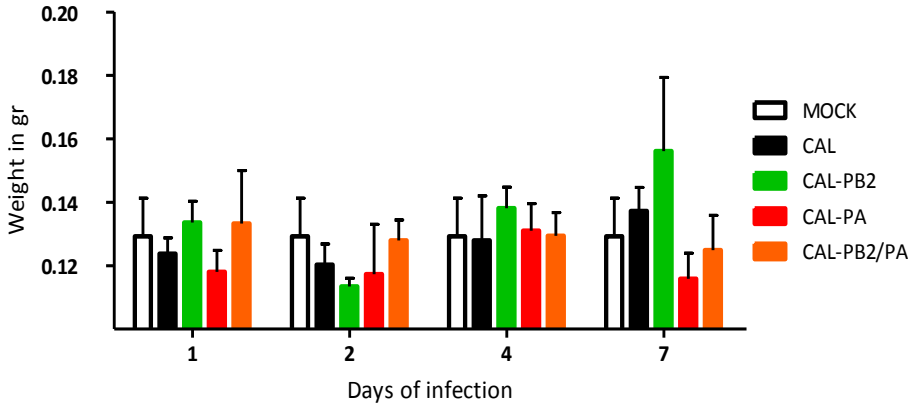
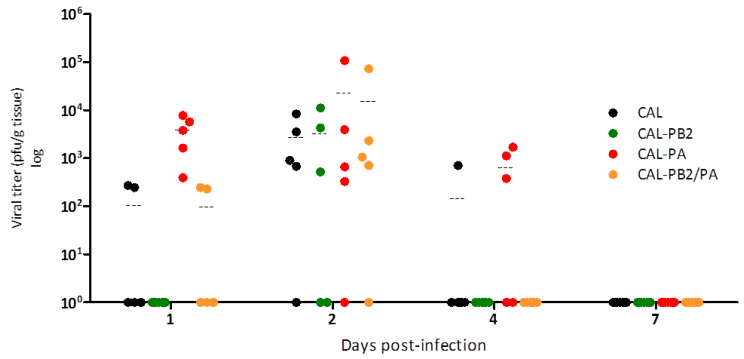


Figure 18. Hearts weight of the mice infected with the different recombinant viruses. Hearts of mock and influenza-infected mice at different time points of infection were excised and weighed; Statistical analysis was performed by unpaired, two tailed Student t-test.

No significant differences in heart weight of infected animals in any group were found suggesting that no severe heart inflammation occurred after infection.

a



b

Virus	1dpi	2dpi	4dpi	Total
CAL	40	80	20	**46,7**
CAL-PB2	0	60	0	**20**
CAL-PA	100	80	60	**80**
CAL-PB2/PA	40	80	0	**40**

Figure 19. Recombinant viruses are able to infect heart tissue. (a) Viral titer in hearts of infected animals. Every point represents viral yield found in each infected animal. Samples without viral particles were given a value of 1. (b) Percentage of infected mice having viral particles in hearts until 4th day of infection.

On 1st day after inoculation all animals infected with CAL-PA virus presented infectious particles in the heart, while 40 % of mice infected with CAL-PB2/PA and CAL recombinants had viral particles in this organ. By the 2nd day, 80% of CAL-PA, CAL-PB2/PA and CAL infected animals, presented infectious particles in their hearts; at this day the attenuated CAL-PB2 virus, reached its maximum value of 60%. The percentage of hearts containing viral particles declined and on the 4th day post-infection, only CAL and CAL-PA infected mice were positive (20% and 60% respectively) to viral presence in heart tissue. By the 7th day all animals were negative for virus detection in the hearts. Having into account the total number of mice having viral particles in the hearts between 1 and 4 days of infection *(Figure 19b)*, 46,7% of CAL-infected mice were positive and this number raises to 80% for CAL-PA infected animals. Therefore, infectious viral particles can be found with higher frequency and at prolonged period of time in mice infected with the high pathogenic CAL-PA recombinant virus.

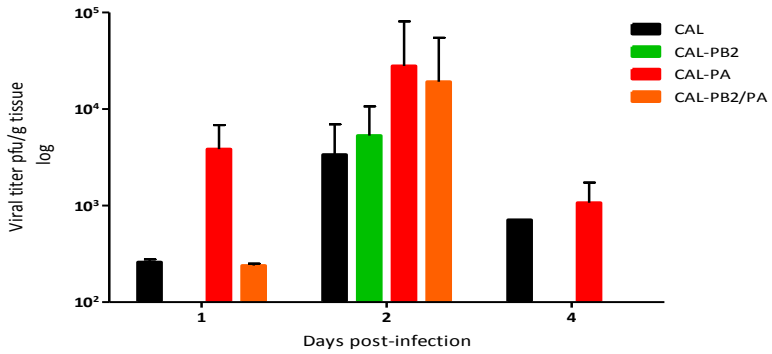


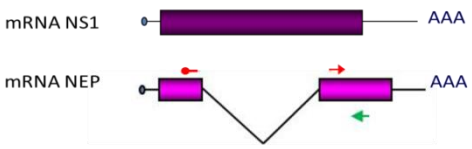
Figure 20. Viral titer in heart samples of animals infected with the different recombinant viruses. Mean and SD of viral titers detected in the hearts of mice infected with CAL wild-type or mutant recombinant viruses. Statistical analysis were performed with unpaired, two tailed Student t-test showed no significance in viral titer in heart samples

Comparing the presence of infectious particles in lung and heart of infected mice, it can be observed a lack of correlation of viral titers in both organs. This can be observed in data from Figure 21b, for instance on the 4th day post-infection there are infectious particles in the lung of all mice *(Figure16)*, whereas only CAL and CAL-PA infected mice have virions in their hearts *(Figure 20)*. Therefore, simple viral transmission is ruled out as the cause of viral detection in hearts. These results suggest that pathogenicity of recombinant viruses carrying PA mutation may be increased by the fact that animals infected with these viruses have a prolong exposure to higher viral titers in hearts than other groups.

Next we examined whether infectious viruses present in the hearts were the consequence of viral replication inside this organ. Although viral titers in lungs do not correspond to viral titers in hearts, it cannot be excluded that viral particles found in heart were due to circulation derived from infected lungs given the proximity of the two organs, and high blood circulation. To analyse if hearts' viral particles were the result of viral replication within this organ we performed a qPCR to detect NEP mRNA (Methods 3.3.1).

The mRNA of NEP is a spliced mRNA transcribed from NS viral segment and it is only present in cells with ongoing viral transcription (Introduction 2.5).

a



b

	Hearts with infective particles				Hearts without infective particles				
2 dpi	CAL	CAL-PB2	CAL-PA	CAL-PB2/PA	MOCK	CAL	CAL-PB2	CAL-PA	CAL-PB2/PA
Viral titer in hearts	$3.6X10^3$	$4.3X10^3$	$1.1X10^5$	$2.3X10^3$	-	-	-	-	-
NEP mRNA	+	+	+	+	-	-	-	-	-
Viral titer in lungs	$2.3x10^6$	$1.4X10^5$	$3.4X10^7$	$2.6X10^6$	-	$1X10^7$	$1.4X10^5$	$8.3X10^7$	$2X10^6$

Figure 21. Recombinant viruses are able to replicate in heart tissue of infected animals. (a) Transcript products from NS viral segment. Arrows depict the primers, forward primer fusion (*red*) and reverse primer (*green*) used for detection of NEP mRNA (b) Viral titers found in hearts and the corresponding viral titer in lungs of same infected animals on the 2dpi. Presence of NEP mRNA in samples with viral titer in hearts and absent in negative samples is shown.

The mRNA of NEP was detected in hearts where infected particles were found, indicating that the recombinant viruses are able to infect and replicate in the heart of infected mice. This characteristic of recombinant viruses may contribute to their pathogenicity especially in the CAL-PA recombinant virus, since animals infected with this virus have virus capable of heart infection at highest titers and at longer period of time than other infected animals.

2.4 Immune response in lungs of infected mice

Virus pathogenicity depends on the nature of the virus, the host conditions and its rapid and adequate response to the infection. To see if these recombinant viruses produced different immune response we monitored innate immune cells influx (neutrophils, alveolar macrophages, monocytes etc.) in infected lungs at early stages of sub lethal infection by flow cytometry analysis (Methods 5.5 and 5.6). For significance analysis of differences between groups we used regular two-way ANOVA statistical analysis with Bonferroni post-test.

- **Neutrophils** are the first cells to be recruited upon viral infection and its influx is crucial for development of diseases and severity of inflammation process.

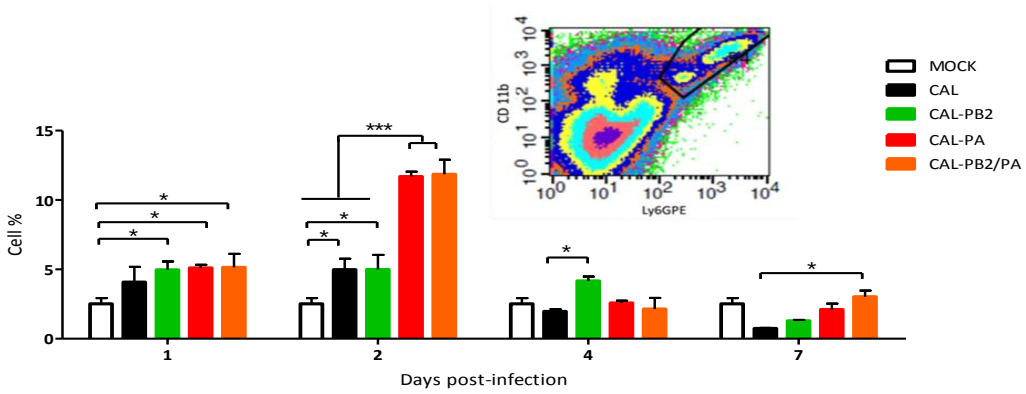


Figure 22. Neutrophils. Influx of neutrophils (cells expressing Ly6GPE^{+}CD11b^{+}CD45^{+} receptors gated in dot plot) into the infected lungs throughout the infection *** p<0.0001, *p<0.05

The neutrophils influx is presented in Figure 22. On the 2nd day of infection influx reaches its maximum levels in recombinant viruses with PA mutation (CAL-PA and CAL-PB2/PA) while infiltration in lungs of animals infected with CAL-PB2 recombinant remains at the same level than control CAL virus. Further on, the only animals that maintain significant influx compared to that infected with control CAL virus on the 4th day, are that infected with CAL-PB2 virus. In summary animals infected with recombinant viruses that have mutations in PA polymerase subunit (CAL-PA and CAL-PB2/PA viruses) cause dramatically high influx of these cells on the 2nd day of infection.

- **Alveolar macrophages** are the first cells to detect pathogens in lungs and are target cells for influenza infection.

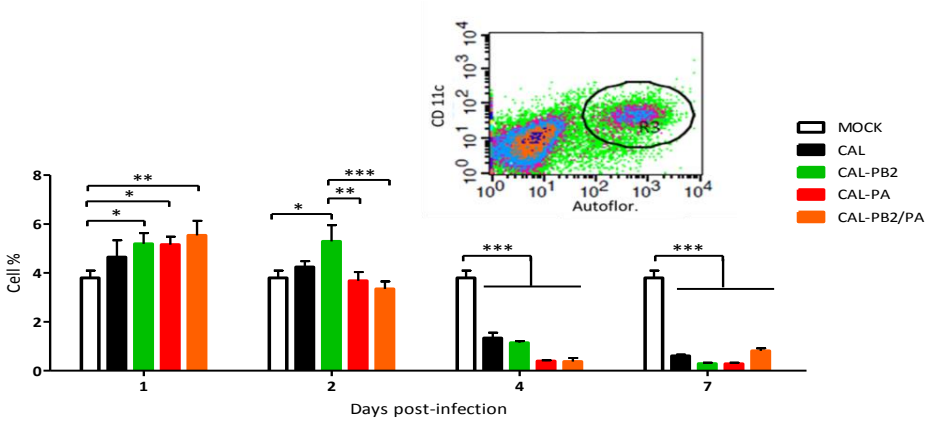


Figure 23. Alveolar macrophages. Influx of alveolar macrophages (cells expressing CD11c^{+}CD45^{+}autoflorescence^{+} gated in dot plot) in the infected upper left lung lobe; Statistical analysis have been done by two-way ANOVA statistical analysis with Bonferroni post-test ***p<0.0001, **p<0.005, *p<0.05

The results of alveolar macrophages influx are presented in Figure 23. There is a significant displacement of alveolar macrophages to the left upper lung lobe in recombinant viruses-infected mice compared to that of the mock-infected animals at the beginning of the infection (1st dpi). On the 2nd day of infection the level of alveolar macrophages in attenuated recombinant virus (CAL-PB2) infected mice remains unchanged, while animals infected with more pathogenic recombinant viruses (CAL, CAL-PA, CAL-PB2/PA) show the same level as the mock infected mice. By the 4th day of infections there are no significant differences in the level of alveolar macrophages in all infected animals. Even though first response of alveolar macrophages to infection is the same, the level of these cells dramatically drops on

the 2nd day of infection in lungs of mice infected with more pathogenic viruses. This may indicate that besides alveolar pneumocytes, alveolar macrophages might be widely infected at the beginning of the infection in these animals, which might delay activation of adaptive immune response increasing the pathogenicity.

- **Dendritic cells (DCs),** besides macrophages, are professional antigen presenting cells, and very important in activation of adequate adaptive immune response in infections. We monitored the influx of conventional DCs in the upper left lung lobe of infected mice at several days post-infection.

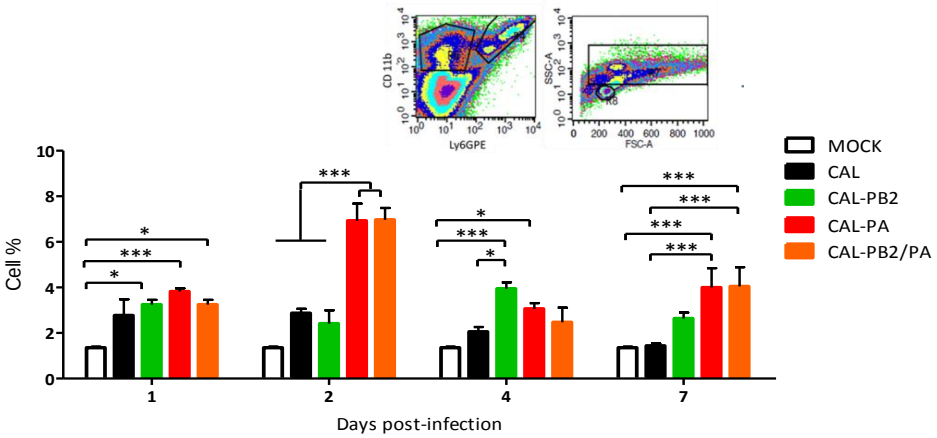


Figure 24. Dendritic Cells. Influx of conventional dendritic cells (cells expressing $CD45^{+}CD11b^{+}$ yet not $CD11c^{-}Ly6GPE^{-}$ selected in the left gate in the left dot plot separated by its size and complexity right dot plot) into the infected lungs; Data were analysed with two-way ANOVA with Bonferroni post-test *** p<0.0001, ** p<0.005, *p<0.05

Similar to alveolar macrophages at the first day of infection we observed significant influx of presumably conventional DC in all virus-infected compared to mock-infected animals, except for CAL infected ones *(Figure 24)*. On the 2nd day of infection influx of DCs in animals infected with recombinant viruses with PA mutation show significantly higher levels of DCs in upper left lung lobe than other infected groups. All throughout infection, mice infected with the recombinant CAL virus showed the same level of conventional DCs as uninfected group. Attenuated recombinant virus (CAL-PB2) provoked significant influx of DCs compared to uninfected group on the 1st and 4th day of infection, which may be due to activation of adequate adaptive immune response in these mice. Even though levels of conventional DC lowers during the infection in these animals they are still highly significant when compared with both uninfected and CAL infected groups. This suggests that infection with both CAL-PA and CAL-PB2/PA recombinants led to higher influx of conventional dendritic cells in upper left lung lobes of infected animals with an utmost peak on the 2nd day of infection.

All virus-infected animals showed influx of DCs; only those infected with the most pathogenic viruses (carrying PA mutation, CAL-PA and CAL-PB2/PA) showed a significantly higher amount of DCs on the 2nd day of infection.

- Monocytes have the ability to differentiate in both macrophages and monocyte derived dendritic cells (mDC) in the ongoing infection. Due to their importance in the immune response, the influx of these cells was analysed in the infected animals. Since, specific antibodies for monocytes or dendritic cells were not used in this experiment, we separated these two cell population by size and complexity.

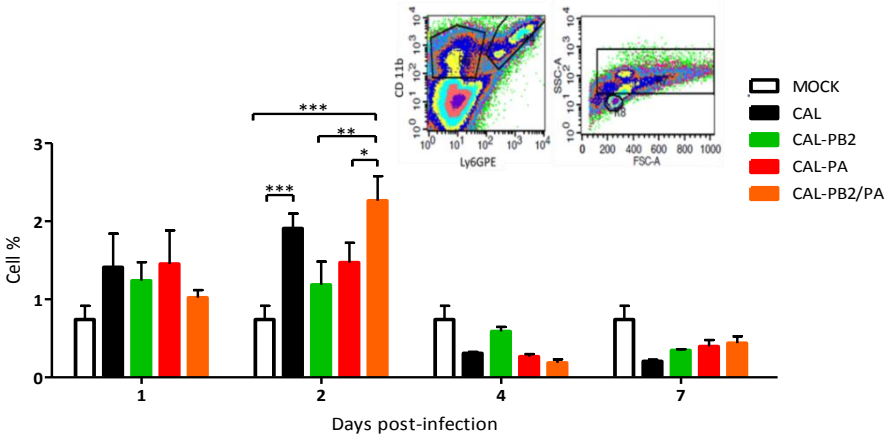


Figure 25. Monocytes. Influx of monocytes (cells expressing $CD11b^{+}$ yet not$CD11c^{-}Ly6GPE^{-}$ selected in the left gate in the left dot plot separated by its size and complexity right dot plot) into the infected lungs; Statistical analysis were performed with two-way ANOVA with Bonferroni post-test ***p<0.0001, **p<0.005, *p<0.05

The results of influx of monocytes are presented in Figure 25. No significant differences were found on the 1st, 4th and 7th day of infection among the mice infected with the different recombinant viruses, whereas significant differences were observed on the 2nd day of infection. The reference recombinant CAL and the CAL-PB2/PA viruses had significantly higher influx of these cells on the 2nd day of infection than other infected groups. Since influx of conventional DCs has not been detected in CAL infected animals, higher level of monocytes might be an attempt to attain normal DC immune response by induced differentiation of monocytes into dendritic cells mDC.

- Inflammation. To study differences in inflammation process we analysed the expression of cytokines and chemokines in mice infected with the reference CAL virus and with the virus with the highest pathogenicity (CAL-PA) at 2dpi (Methods 3.4). The results are presented in Table 8.

Gene	Fold Change CAL-PA vs CAL	P value
Ccl3	3.3	0.011
Ccl4	3.8	0.006
Cxcl13	2.9	0.016
Cxcl9	4.7	0.002
Cxcl10	3.5	0.006
Ccl7	3.7	0.005
Ccl2	4.0	0.004
Ackr1	2.9	0.039
Cxcl11	3.8	0.005
Ccl19	4.7	0.022
Ccr5	2.8	0.017
Ly6g (lymphocyte)	6.1	0.015
Csf3	7.2	0.001
Il6	2.23	0.040
Saa 1(serum amyloid)	7.4	0.001
Saa 2	4.8	0.019
Saa 4	10.1	0.001

Table 8. Inflammation process in infected mice lungs . Expression of pro-inflammatory cytokines and chemokines in infected lung tissue with CAL and CAL-PA viruses; significance analysis was performed with FIESTA.

Chemokines responsible for infiltration of monocytes (MCP1, MCP3, Cxcl10), macrophages (MIP-1α, MIP-1β), neutrophils (Csf3), T cells (Ccl19, Cxcl9, Cxcl10, Cxcl11) and B cells (Ccl19, Cxcl13) are overexpressed in lung tissue of CAL-PA compared to CAL infected animals *(Table 8)*. Overexpression of Ly6G in CAL-PA infected lungs indicates high infiltration, recruitment and migration of neutrophils. Ccr5 overexpression (CD195) in T cells, DC and macrophages of CAL-PA infected animal indicates acute inflammatory state in infected lung. All these data correlate with flow cytometry study and prove higher inflammation process in CAL-PA compared to CAL infected mice. Higher expression of serum amyloid A genes imply severe inflammation in infected lungs since these proteins have been conferred as biomarkers of acute inflammation in patients with chronic obstructive pulmonary disease *(Calero, Arellano et al. 2014, Sun and Ye 2016)*.

3. Antiviral response in human infected cells

Recombinant viruses induce different innate immune response, which strictly correlates with their previously determined pathogenicity and mortality *in vivo*. CAL-PA and CAL-PB2/PA recombinant viruses that were the most pathogenic in the mice model were also able to induce higher innate immune response, higher titers in both lungs and hearts, and stronger influx of immune cells and inflammation that may contribute to its overall pathogenicity.

To examine if the same outcome can be achieved in infections of human cells we infected human alveolar A549 cells with the different recombinant viruses and with an influenza virus lacking NS1, a viral protein mainly involved in counteract immune response, as control virus *(Garcia-Sastre, Egorov et al. 1998)*. The induction of antiviral genes and accumulation of viral proteins were analysed 16 and 24 hours post-infection (Methods 3.3.1 and 4.2).

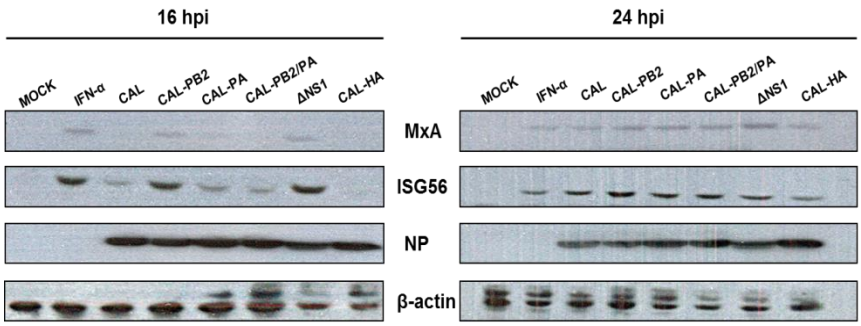


Figure 26. Accumulation of antiviral proteins MxA and ISG56 and viral NP protein. Cultured A549 cells were infected at 0.5 moi with the recombinant viruses. At the indicated hpi, samples were used to detect the indicated proteins by Western blot. MOCK; cells treated with PBS as negative control. ΔNS1; cells infected with a virus lacking NS1 protein *(Garcia-Sastre, Egorov et al. 1998)* IFN; cells treated with 1000u/ml of IFNα2 as positive controls

The results show that accumulation of ISG56 and MxA 16 hours after infection is clearly detectable in cells infected with the low pathogenic virus CAL-PB2, while cells infected with CAL-PA, CAL-PB2/PA and CAL viruses have less accumulation of these proteins. CAL-HA infection induces expression of ISG56 and MxA later than any other recombinant even though it has a similar replication rate compared with the others recombinants. In the *in vitro*

infection, with those recombinant viruses that were more pathogenic in mice, there is a significant subtle delay in ISG56 accumulation compared with the infection with attenuated CAL-PB2 virus and positive controls *(Figure 26)*.

To further evaluate the slight differences observed in the accumulation of antiviral proteins, additional experiments were performed analysing the expression of the corresponding genes in A549 respiratory cells by qPCR. The mRNAs for MxA and ISG56 genes were determined in the cells infected with the different recombinant viruses. Additionally, to evaluate the correlation of the induction of these genes with their putative inducer, levels of IFN-β gene were also determinate in this same assay.

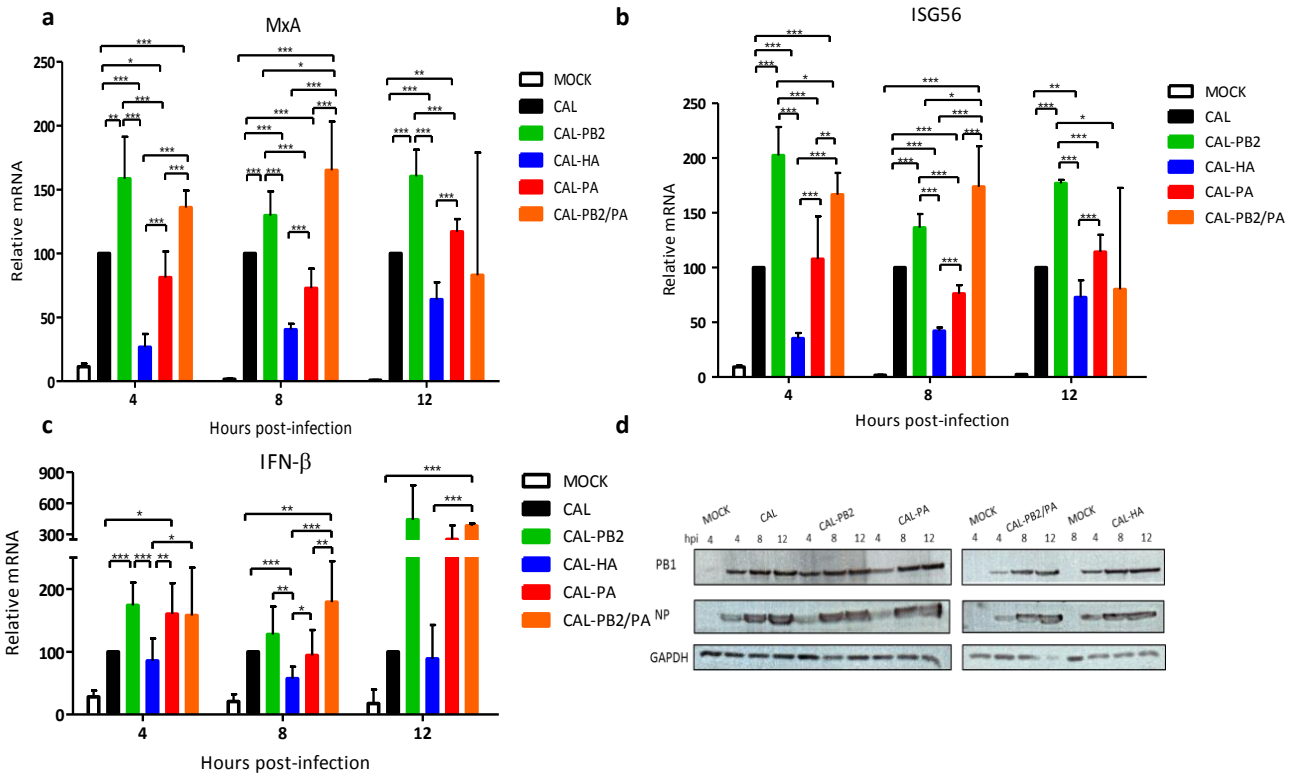


Figure 27. Relative amount of the indicated mRNAs in A549 cells infected at moi 0.5 with the different recombinant viruses. Expression of (a) MxA, (b) ISG56 and (c) IFN-β genes during the first 12 hours of infection. The values obtained in CAL-infected cells are taken as 100% and the amount of rRNA28S was used to normalize every determination. (d) Control of kinetics of infection for every recombinant viruses in A549 cells performed by WB against the indicated proteins 4,8,12 hours post-infection. Statistical data were analysed by unpaired, two tailed Student t-test ***p<0.0001, **p<0.005, *p<0.05

The quantitative data *(Figure 27)* correlate with the results obtained for protein accumulation 16h after infection showed in *(Figure 26)*. CAL-HA recombinant does not induce antiviral response efficiently as the other viruses do. Recombinant CAL-PB2 virus induces the highest expression of antiviral genes, while interestingly, previously described more pathogenic CAL-PA virus induces less antiviral response than CAL-PB2. PA mutation is able to reduce the activation of antiviral response of the CAL-PB2, as it is shown by recombinant virus bearing both PA and PB2 mutations (CAL-PB2/PA; F-like-polymerase virus). Although at the beginning of infection induction of IFN-β in cells infected with CAL-PA virus is higher than that of CAL virus, the same level of induction is observed at later time of infection (8 and 12 hpi). Induction of MxA and ISG56 is significantly lower before the end of the first viral cycle in CAL-PA virus infected cells. This is observed 4 and 8 hours upon infection in the case of MxA gene and 8 hpi in the case of ISG56 gene compared with our CAL control *(Figure 27)*. This suggests that previously described more pathogenic recombinant CAL-PA virus induces less antiviral response in human pneumocytes than other recombinant viruses in early stage of infection, with an exception of CAL-HA virus that does not induce antiviral response at all, even though all of these viruses replicate at the similar level in the first 12 hours of infection *(Figure 27d)*.

4. Role of defective genomes (DGs) in viral pathogenicity

Interesting results from previous experiments indicated that CAL-PA recombinant keeps the same level of replication than the other recombinant viruses in A549 infected cell and yet restrains antiviral response of these cells. A possible explication for this characteristic of CAL-PA virus could be that its polymerase, even though does not possess higher activity than that of the others recombinant viruses, has higher fidelity and/or stability of the complex itself. This would lead to fewer errors during replication process, lower production of defective genomes (DGs) and partial inhibition of antiviral response in infected cells. To address this possibility we analysed DGs generation of all recombinant viruses.

Production of DGs was previously detected in both cell culture and mice infected with F and M strains. Deep sequencing showed that M strain generates more than 7 fold higher amounts of DGs than F strain. Furthermore, although not quantitative, simple PCR analysis indicated that ratio between full-length viral genome and DGs in both PB2 and PA segments was higher in the lung samples of more pathogenic F virus infected mice than in M-infected mice (manuscript under review). Since, production of DGs is a by-product of replication; viral polymerase plays a key role in its generation. Therefore, changes detected in the viral polymerase subunits of F strain may contribute to lower DGs production.

To investigate this possibility we performed deep sequencing analysis. Recombinant viruses were grown in cell culture, viral particles were purified from cell supernatants followed by RNA isolation that was used for deep sequencing using the same criteria for defining DGs applied in F and M isolates (Methods 3.4).

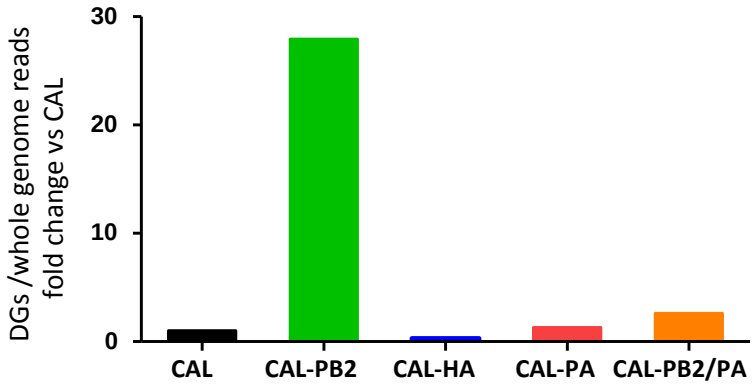


Figure 28. Defective genomes in purified viral particles of recombinant viruses. Recombinant viruses were grown in MDCK cells at 10^{-5} moi and collected after 36 hours of infection. DGs ratios are calculated as jumping reads per million (RPM) that align the viral genome compared with entire genomes, analysed in purified virions of recombinant viruses. DGs ratio of the CAL recombinant virus is taken as 1 and fold change is shown for the mutant viruses versus CAL.

The data showed that CAL virus accumulates low DGs levels, which coincides with the reported high virulence of the CAL reference strain *(Itoh, Shinya et al. 2009)*, and is similar to the F virus. The CAL-PB2/PA and CAL-PA viruses showed DGs accumulation similar to CAL virus (2.6- and 1.3-fold change, respectively; *Figure 28*), whereas CAL-PB2 had a 27-fold higher DGs ratio than CAL virus, which was almost 11-fold higher than CAL-PB2/PA (with the F-like polymerase). These data indicated that the PB2 A221T mutation enabled high DGs accumulation (*Figure 28*; compare CAL vs CAL-PB2), whereas PA D529N mutation restricted their accumulation (*Figure 28*; compare CAL-PB2/PA vs CAL-PB2). The combination of PA and PB2 changes, which mimics F virus polymerase, leads to low DGs accumulation, as also observed for the F virus itself *(Figure 7)*.

We also determined DGs distribution per viral segment for all the recombinant viruses *(Figure 29)*.

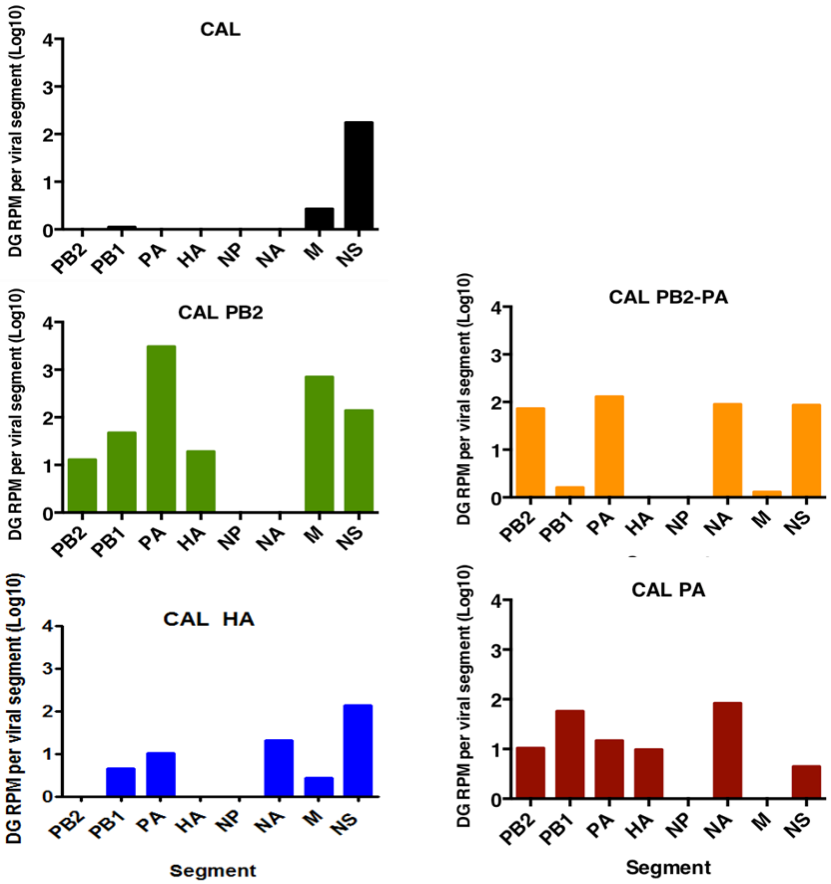


Figure 29. Distribution of generated DGs per viral segment of recombinant viruses. DGs for each viral segment in the recombinant viruses is calculated as jumping RPM that align each viral RNA segment.

We found that DGs distribution per viral segment was random in the majority of the viruses. Although the majority of DGs are produced in the polymerase segments (PB1, PB2 and PA), as previously described, certain viruses also generate large numbers of DGs in other segments *(Figure 29)*. This is particularly observed in CAL virus, which generates DGs mainly in the NS segment, for reasons that remain unclear. This suggests that not just quantity but origin of generated DGs can play an important role in influenza virus infections and its outcome. Nevertheless, CAL-PA recombinant virus retained the F strain phenotype producing a low DGs production capability.

Accumulation of DGs has been described in *in vivo* viral infections *(Saira, Lin et al. 2013)*. To test whether the distinct capacities of these viruses to accumulate DGs could be observed in vivo, we analysed DGs and viral genome accumulation in the lungs of recombinant viruses-infected mice on the 2nd dpi by PCR analysis (Methods 3.3.2). For that two separate PCR reactions were made; one for detection of defective genomes and other that detects an internal fragment of the entire viral gene segment that is absent in DGs. Since defective genomes originate vastly from polymerase genes (PB1, PB2 and PA) we focused on PB2 and PA segments and their DGs.

Similarly to cell culture infection, in *in vivo* infection the recombinant viruses generated defective genomes from PB2 and PA segments and packed them within the viral particles. Although this is not a quantitative technique, the ratios between amplified internal segments and DGs, suggest that CAL and CAL-PA recombinants produce lower amounts of DGs from PB2 and PA segments than the other recombinants in *in vivo* infection.

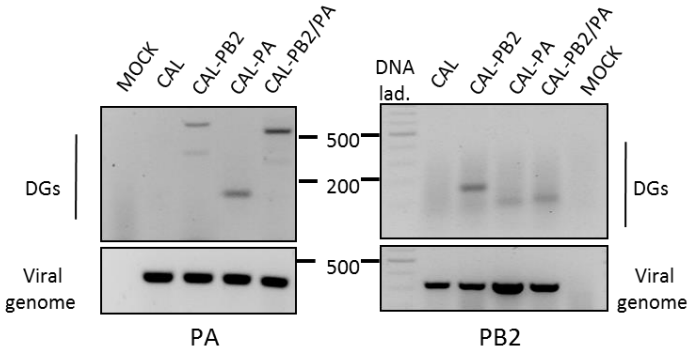


Figure 30. Detection of DGs and internal fragments from full length PB2 and PA genome segments in virions isolated from lungs of infected animals.

To confirm that PA change found in F virus plays a major role on the ability to reduce the accumulation of DGs and that this behaviour contributes to pathogenicity, we took advantage of previously described mutations in matrix 1 (M1) and matrix 2 (M2) viral genes enabling influenza virus to accumulate large amount of DGs *(Perez-Cidoncha, Killip et al. 2014)*. Thus, recombinant CAL viruses carrying these M1/M2 mutations in the presence or absence of PA change were generated (CAL-M and CAL-M-PA viruses, respectively). Deep-sequencing of RNA obtained from CAL-M and CAL-M-PA purified virions was performed. A forty four-fold increase of DGs in CAL-M compared to CAL virus was observed *(Figure 31)* in accordance with the previously described capacity of these changes to increase DGs in an H3N2 influenza virus context. The change D529N in PA gene (CAL-M-PA virus) decreased the DGs ratio of the parental virus *(Figure 31)*.

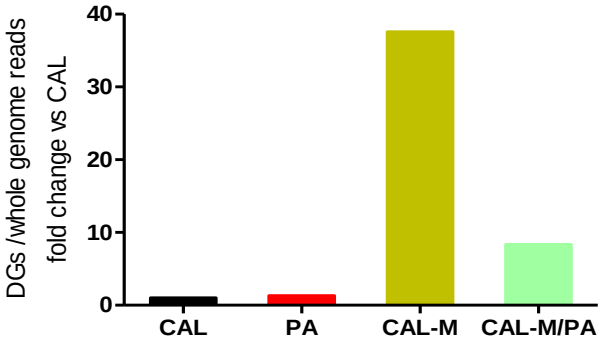


Figure 31. Production of DGs in recombinant viruses carrying mutations in the M and the PA segments. Recombinant viruses CAL-M and CAL-M/PA were grown in MDCK cell line at 10^{-5} moi for 36 hours. The DGs ratios are calculated as jumping RPM that align the viral genome, analysed in purified virions of recombinant viruses. DGs ratio of the CAL recombinant virus is taken as 1 and fold change is shown for the mutant viruses versus CAL.

The DGs distribution per viral segment for these viruses is shown in Figure 32. Introduction of PA mutation in CAL-M virus drops production of DGs especially in M, PA and NA segments. CAL-PA, CAL-M and CAL-M-PA recombinants generated low amounts of DGs from NS segment in contrast to CAL reference virus. These data suggest that the origin of some DGs may play a role in phenotypic manifestations.

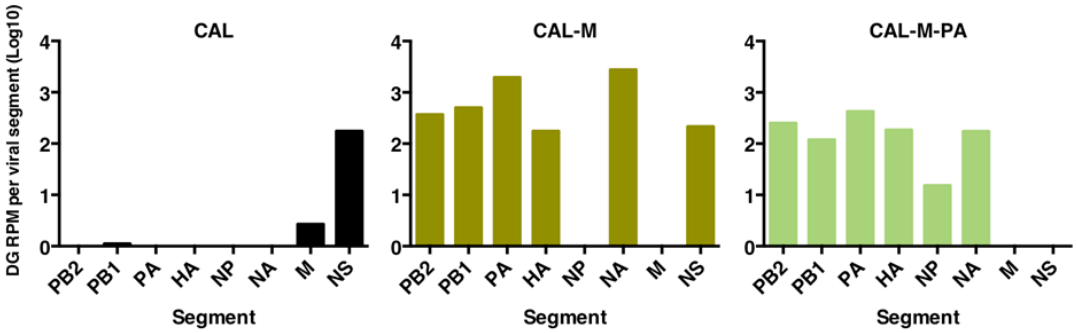


Figure 32. Distribution of generated DGs per viral segment of Cal-M and CAL-M/PA recombinant viruses. Viral particles from each recombinant virus were purified from viral stocks generated at moi 10^{-5} pfu/cell after 36 hours of infection. DGs for each viral segment in the recombinant viruses in Fig.29, is calculated as jumping RPM that align each viral RNA segment.

As shown in Figure 31, introduction of PA D529N mutation in CAL-M virus increases production of DGs. To examine whether accumulation of DGs modifies the pathogenicity of recombinant viruses carrying M and both M and PA mutation, the pathogenicity of the CAL-M and CAL-M-PA recombinants was studied by estimation of LD_{50} in *in vivo* experiment *(Figure 33a)*.

a

Virus	LD_{50}
CAL	$1{,}6x10^5$
M	$> 5x10^5$
M-PA	$> 5x10^5$

b

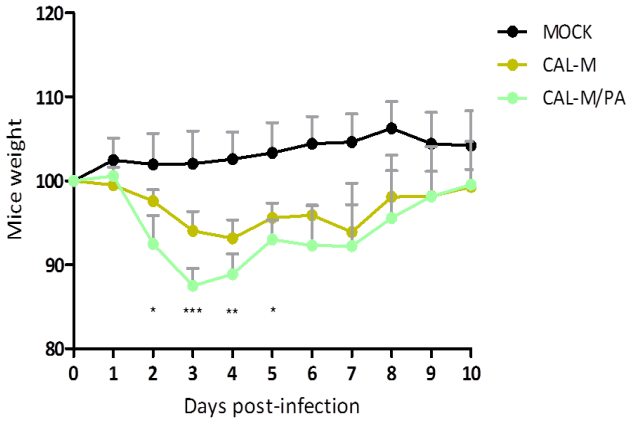


Figure 33. Pathogenicity of CAL-M and CAL-M/PA in vivo.(a) Mice (n = 5) were inoculated intranasally with $5x10^3$, $5x10^4$ and $5x10^5$ pfu of CAL-M or CAL-M-PA. The precise LD_{50} could not be determined and is represented as > the highest dose used. The LD_{50} for CAL recombinant is derived from data in *Table 1*. (b) Mice (n = 5) were inoculated intranasally with a sub lethal dose (5×10^5 pfu) of each recombinant virus or PBS (MOCK) as control. Body weights were determined daily for 10 days and are shown as the percentage of body weight at inoculation (time 0). Significance was determined by unpaired, Student's t test (* p <0.05, ** p <0.01, *** p <0.001).

Introduction of the M1+M2 mutations on the CAL or CAL-PA backgrounds led to clear virus attenuation in mice, as the LD_{50} increased from 1.6 x 10^5 or 3 x 10^3, respectively, to >5 x 10^5 in both cases *(Figure 33a)*. An accurate LD_{50} could not be calculated for both CAL-M and CAL-M/PA recombinant viruses, since they did not produce 50% survival drop at any viral dose that was used. *In vivo* infections with higher viral doses were impossible due to the experimental limitation. CAL-M-PA virus pathogenicity was greater than that of CAL-M virus, as weight loss in CAL-M/PA infected group is significantly lower in the early stages of infection (2^{nd}-5^{th}dpi) after sub lethal infection *(Figure 33b)*. This suggest that PA mutation is a pathogenicity marker in *in vivo* infections, causing more serious signs of illness in infected animals, while M mutation attenuates its pathogenicity by extensively decreasing its $LD_{50.}$ These data support the idea that reduced DGs accumulation is a pathogenic determinant for influenza virus in mice, and that a mutated PA D529N derived from a fatal IAV case decreases DGs generation.

In summary, all these results show that PA D529N mutation does not affect polymerase activity or viral replication in cell culture, but decreases the accumulation of DGs. This mutation increases pathogenicity of the reference strain CAL in animal model; causing 50% of mortality in infected animals in a 100–fold lower dose than control CAL virus and more than 1000-fold lower than original F strain. The increased pathogenicity conferred by PA D529N mutation is mediated by the contribution of several processes, including weak induction of antiviral response, weak production of defective genomes, high replication in the lungs, high influx of neutrophils and dendritic cells, acute inflammation in lungs and high incidence and replication in the hearts with possibly associated cardiac disorders. Altogether, PA D529N change might be responsible for the increased pathogenicity of F clinical isolate from a deceased patient.

Influenza A virus (IAV) seasonal outbreaks and rare but severe IAV pandemics have great impact on human health and global economy. Vaccination is the best prevention and protection from seasonal viral infection. However, a vaccine inducing broad or universal protection from flu infection is still not available *(Krammer and Palese 2015)*. In order to provide helpful knowledge to fight against the constant treat of emergence of highly pathogenic antiviral resistant subtypes of influenza A viruses, it is essential to determine features that makes some influenza A virus particularly virulent.

Pathogenicity and virulence of any viral strain depends on the interplay between several features. Changes in the viral genome have been associated with virulence, pathogenicity and host range factor. Some specific changes lead to adaptation of avian strains to infect and replicate in mammalian cells as well as to increase the overall pathogenicity. Several of these adaptive changes have been detected in the superficial glycoprotein HA; Q226L and G228S in H2 and H3 strains and E190D, S137N and G225E in H1 strains *(Rogers, Paulson et al. 1983, Matrosovich, Tuzikov et al. 2000, Ni, Kondrashkina et al. 2015)*. Others have been described in the other viral genes such as E627K *(Subbarao, London et al. 1993)*, D701N, S714I/R *(Czudai-Matwich, Otte et al. 2014)* and T588I *(Zhao, Yi et al. 2014)* in PB2 polymerase subunit, N375S *(Taubenberger, Reid et al. 2005)* in PB1 protein, N66S *(Mazur, Anhlan et al. 2008)* in PB1-F2 and T85I, G186S and L336M *(Bussey, Desmet et al. 2011)* in PA subunit. Some changes in viral genome that result in accumulation of defective genomes (DGs) during replication *(Fodor, Mingay et al. 2003)*, decrease the pathogenicity of viruses by inducing early host cells antiviral response *(Tapia, Kim et al. 2013)*.

These reports underline the importance of interplay of both viral and host factors in pathogenesis of influenza virus infection. Besides genetic viral background, host response to viral infection is equally important for the pathogenic phenotype of the viral strain. Appropriate and adequate immune response will prevent the viral infection disabling its further viral spread and controlling inflammatory process avoiding unnecessary tissue damage and injury. In this context, we have identified and characterized an influenza virus marker involved in high replication, but also in increased induction of innate immune response to viral infection and enhanced viral spread in murine model. All of these effects together contribute to high pathogenicity of virus and increased mortality rate in infected animals. These findings provide an insight into complex and multifactorial process that makes influenza A virus highly pathogenic.

1. Pathogenesis of influenza virus

During the 2009 influenza pandemic, it was observed that infection with the new A(H1N1)pdm09 viruses produced mild symptoms in the majority of infected patients, but compared with the previous seasonal H1N1 cases, they caused a greater rate of severe or complicated illness in healthy young adults and children *(Louie, Acosta et al. 2009, Perez-Padilla, de la Rosa-Zamboni et al. 2009)*. The co-morbid conditions were similar for both the pandemic A(H1N1)pdm09 and previous seasonal influenza viruses and include chronic metabolic disease, primarily diabetes mellitus and renal disease, chronic lung and cardiac disease, immunosuppressive conditions and neoplasms *(Hlavinkova, Kristufkova et al. 2015)*. In addition, obesity and pregnancy were associated to A(H1N1)pdm09 severe infections *(Falagas, Koletsi et al. 2011, Singanayagam, Singanayagam et al. 2011)*. The pre-existing immune status and the existence of underlying chronic conditions definitely contributed to the disease outcome. However, some of the infected patients developed severe symptoms without any obvious impairment in health condition. Therefore, we hypothesized that different viral

strains during 2009 pandemics were co-circulating in human population with different pathogenicity risk to human health *(Rodriguez, Falcon et al. 2013)*.

To analyse possible virulence differences, our group compared two contemporary human A(H1N1)pdm09 viruses from the first wave of 2009 pandemic isolated from two patients without known co-morbid conditions and aged under 65, one from a patient with the fatal outcome (F) while the other showed only mild respiratory disease (M). Comparison of the genome of these two clinical isolates identified several changes in the virus isolated from the fatal case patient that have never been associated with any increased pathogenicity in Influenza A nor B viruses. These changes were present in the polymerase subunits (PB2 A221T and PA D529N) and the hemagglutinin (HA S127L) *(Rodriguez, Falcon et al. 2013)*. Further characterization has demonstrated that none of the polymerase changes affects viral replication *(Figure 10)* or viral polymerase activity *(Figure 11)* in ongoing viral infection in human alveolar epithelial cells (A549). Pablo López in our laboratory has evaluated that the antigenic properties of HA127L are similar to wild type HA. Additionally, it has been shown that the entrance of vRNPs in CAL-HA127L infected cells is similar, or at least not faster, than CAL infected cells (data not shown). These data suggest that HA127L mutation may not alter the main activities attributed to this protein, such as the antigenic properties and viral entry in the cell.

1.1. Identification of the main driving mutation of a fatal human virus pathogenesis

Pathogenicity of viruses carrying the selected mutations found in F virus has been extensively evaluated. We have characterized the morbidity and mortality caused by recombinant viruses carrying single PB2 A221T, PA D529N and HA S127L changes in their genome, as well as combinations of them *(Table 6)*. Unexpectedly, recombinant viruses with HA S127L mutation alone (CAL-HA) or accompanied with any other change showed no pathogenic trait and were attenuated compared to the control CAL virus, even though they showed tendency of reaching higher viral titer in cell cultures than the other recombinant viruses *(Figure 10).* Growth kinetics of recombinant viruses in 3T3 mouse cells revealed that all recombinant viruses display a similar grow rate (data not shown), while the CAL-HA virus showed a tendency to have lower viral titers than the other viruses in the first 48 hours of infection. Mutation HA S127L is positioned proximal to one of the three secondary structure that together form functional receptor binding site of HA, 130 loop *(Ping, Keleta et al. 2011, Lu, Qi et al. 2013)*, therefore it may be involved in the recognition of the viral receptor constituting a host range factor, supporting a lower production of infective particles in mouse cells infected with this virus. Recombinant viruses with PB2 A221T mutation (CAL-PB2) in polymerase subunit were also attenuated compared to parental CAL virus and showed no pathogenic feature in infected animals, except when it was accompanied with PA D529N mutation (CAL-PB2/PA). Therefore, mutations HA S127L and PB2 A221T do not seem to cooperate to the high pathogenicity of the F virus and in fact they may act as attenuation changes.

Changes in PA subunit affect pathogenicity and virulence of influenza A clinical strains from avian origin *(Yamayoshi, Yamada et al. 2014)*, moreover in 2009 pandemic strains a key role for increased pathogenicity was found in the PA subunit of viral polymerase *(Song, Pascua et al. 2011)*. Recombinant virus with introduced PA D529N mutation (CAL-PA) showed increased pathogenicity in murine model lowering the viral doses that causes 50% of mortality (LD_{50}) *(Table 6)* by more than 300 fold compared to the parental clinical strain isolated from a deceased patient (F). This CAL-PA feature is diminished by introduction of any other of the two selected changes found in the F lethal strain.

Therefore, PA D529N change is solely responsible for increased pathogenicity of recombinant CAL-PA and CAL-PA/PB2 viruses in murine model. It is not unusual that one mutation in viral polymerase may increase the pathogenicity of recombinant strains, but this particular mutation has never before been associated with any increased virulence or pathogenicity. Furthermore, PA D529N change has been observed very scarcely among the pH1N1 strains, human seasonal strains, avian and swine viruses. In fact the total calculated frequency of appearance of PA D529N change in the influenza viruses present in these host is 0.048%, analysed using the influenza virus resource databases from NCBI. Cristal structure of the C-terminal domain of PA subunit revealed that several amino acids implicated in the replication activity of the polymerase complex (E 524, K 536, W 537) *(Obayashi, Yoshida et al. 2008)*, are neighbouring the 529 amino acid. This amino acid is located just in front of 3_{10} helix, an α helix that possesses 3 amino acids (P530-R531-L532) per turn. The 3_{10} helix is a secondary structure, extension of α- helixes usually found in terminals of proteins or polypeptides. This 3_{10} helix structures are involved in helix-coil transitions *(Armen, Alonso et al. 2003)*. Aspartic acid (D), at N0 position, just in front of the 3_{10} helix, stabilizes 3_{10} helix structure *(Karpen, de Haseth et al. 1992)*, therefore D529N mutation may destabilize 3_{10} helix and affect helix–coil transitions of C-terminal PA domain. This helix transition may affect overall stability and transition from transcription to replication process of viral polymerase.

Influenza A virus polymerase exists in different oligomerization state *(Jorba, Area et al. 2008, Moeller, Kirchdoerfer et al. 2012)*, which allows different assembly of polymerase monomers during replication *(Jorba, Coloma et al. 2009)*. New mechanism has been proposed for the transition of influenza virus from transcription to replication, suggesting that instead of one viral polymerase bound to vRNP two molecules are involved in this process. The proposed mechanism suggests that the RNA depending RNA polymerase (vRdRP) dimer bound to viral RNA recruits another free polymerase dimer to form transient tetramer, which initiates replication of viral genome *(Chang, Sun et al. 2015)*. This model presumes that thumb domain of PB1 is seated on top of C-terminus of PA (PA-C) subunit, which forms the RNA binding and RNA polymerization active site. PA-C/PB1 interaction emphasizes the key role of the highly conserved charged residues in PA-C domain; K539, R566, and K574 essential for RNA binding and synthesis of all three types of RNA (vRNA, cRNA and mRNA), which are closely positioned to our 529 residue and 3_{10} helix. Residues in α5, α6, α7, α8 helixes, in the same domain as 3_{10} helix of PA protein, are important for RNA binding, but also for PB1 interaction (E427, I428, R442, R443, R582, L586, Q590, Q591, and E593, respectively) in the tetramer sub complex *(Chang, Sun et al. 2015)*. In the tetrameric structure, PB1 C terminus and PB2-N terminus are located away from the active site cavity, in contrast to proposed monomer state RNA synthesis mechanism *(Reich, Guilligay et al. 2014)*. Mutation R638 in PA subunit that attenuates viral growth and induces production of defective genome was found in PA-C terminus domain *(Fodor, Mingay et al. 2003)*. C453 and R638 mutations, both in PA-C terminus domain, destabilize RNA-PA subunit interaction (*Fodor, Mingay et al. 2003)* underlying the significance of PA-C domain in intact genome viral replication.

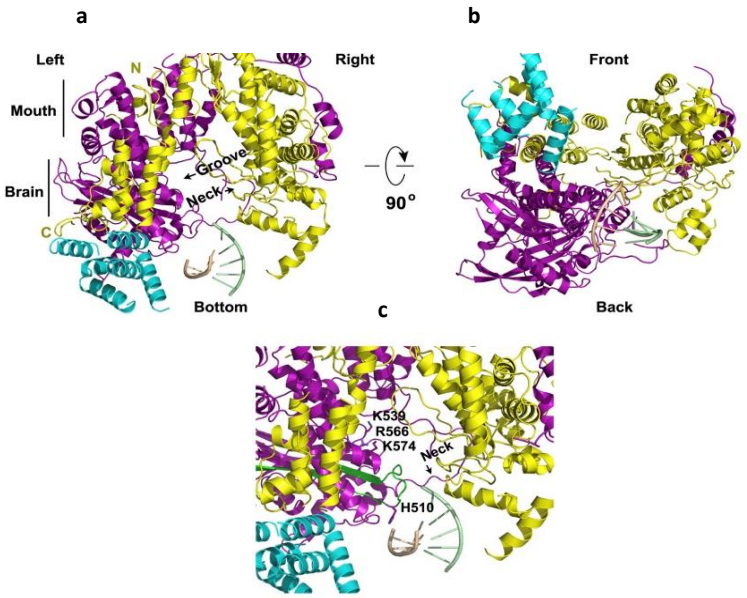


Figure 34. PA-C and PB1 catalytic domain in tetrameric state of polymerase complex. (a, b) PA-C domain (purple) and PB1 catalytic domain (yellow) presented from different perspective, unassigned helixes are presented in blue; predicted PA-C domain (c) indicate β-6 and β-7 barrel (green) as critical features for RNA (light green and brown) binding. 3_{10} helix and D529N are 10 aa prior to the β-6 barrel. *(Shenghai Changatall at all. Molecular Cell, Issue5, 2015, 925–935)* **http://dx.doi.org/10.1016/j.molcel.2014.12.031**

1.2 Role of defective genomes (DGs) on viral pathogenesis

Different RNA viruses produce defective genomes (DGs) as a direct product of viral replication, and their error prone polymerase. DGs have been detected in hospitalized patients infected with respiratory syncytial virus (RSV) and moreover their amount in clinical samples and activation of cellular antiviral response were positively correlated *(Sun, Jain et al. 2015)*. Viral RNA replication and generation of DGs depend largely on viral polymerase and therefore single mutation in any of the subunits of the heterotrimeric influenza virus polymerase complex may affect its production. Our findings show that *in vivo* attenuated recombinant CAL-PB2 virus showed excessive production of DGs in cell culture, while more pathogenic recombinant viruses accumulated lower amounts of truncated viral RNAs *in vitro (Figure 28)*. Both recombinant viruses with PA mutation (CAL-PA, CAL-PB2/PA) accumulated a reduced amount of DGs, which positively correlates with its pathogenicity in mice. However, the CAL-PB2/PA virus (with the F-like polymerase) generated twice as much defective vRNAs than CAL-PA virus which may indicate that influenza virus polymerase carrying PB2 A221T mutation generates excess of defective genomes during the infection and PA is able to partially decreased its production. This intermediate accumulation level of DGs in PAL-PB2/PA virus correlates with an LD_{50} between CAL–PA and CAL-PB2 viruses.

The control, CAL recombinant virus showed a very low accumulation of DGs in virions isolated from infected cells, which coincides with the reported high virulence of the CAL reference strain *(Neumann et al., 2009)*, and is similar to the F virus *(Figure 7 and 28)*. However, CAL was less pathogenic than CAL-PA recombinant in mice, suggesting that additional features to the total amount of DGs are involved in the different pathogenesis of these viruses. While most

of the DGs generated by recombinant viruses carrying mutations in the viral polymerase were originated from PB1, PB2 and PA gene segments, as previously described *(Dimmock and Easton 2014)*, the majority of DGs in CAL virions were originated from NS segment *(Figure 29)*. This rare and exclusive distribution of DGs was similar in CAL-HA recombinant virus, which possesses an identical polymerase to that of CAL virus. Further analysis showed that the DGs from NS segment would synthesize a positive ssRNA with a deletion that would produce a NS1 protein without the RNA binding domain. Such a defective NS1 protein with impaired ability to counteract the antiviral response would decrease the virulence of these viruses *(Garcia-Sastre, Egorov et al. 1998)*. The distribution of DGs per segment has been evaluated in virions isolated from infected cells; in more complex model such as infected mice this effect could be even more accentuated, exacerbating the decreased pathogenicity of these recombinants. Mice infected with these viruses would have lower viral titers in the lungs *(Figure 16)* due to stronger antiviral response which would lead to decreased inflammation processes in infected tissue, as we observed in our work *(Table 8)*. Pathogenicity of the recombinants that do not possess mutations in viral polymerase (CAL and CAL-HA) correlates with this assumption. Since the high throughput sequencing has been performed in purified virions where viral mRNAs are not present, it is still unknown whether the positive ssRNA is actually transcribed from DG of the NS segment during infection and further experiments will be performed to evaluate this interesting hypothesis. Our observations suggest that not only quantity but also quality of generated DGs may affect the overall pathogenicity of influenza A viruses.

1.2.1. Effect of PA D529N mutation on defective genomes accumulation during the infection

The reference CAL/04/09 virus is one of the first, highly pathogenic clinical isolates of the 2009 pandemic outbreak *(Itoh, Shinya et al. 2009, Swiss Institute of Bioinformatics)*. As mentioned, this virus has a low capacity of DGs generation, thus estimation of additional reduction of DGs generation by the introduction of a single PA D529N mutation in viral polymerase seems to be problematic. To circumvent this problem and have an experimental model that allows the examination of the role of the D529N mutation in PA polymerase subunit on DGs production *in vitro,* we used recombinant viruses with mutations in M viral segment that increase accumulation of DGs during the infection *(Perez-Cidoncha, Killip et al. 2014)*. Recombinant viruses carrying mutations in M segment (CAL-M) and in both M and PA segments (CAL-M/PA) generate high amounts of DGs *(Figure 31)*. Interestingly, recombinant CAL-M/PA virus, with M and additional PA D529N change, accumulated 4.6 folds less amount of DGs than the CAL-M recombinant. This data implies that D529N mutation in PA subunit decreases DGs generation in recombinant viruses with a high DGs generation capacity.

PA D529N mutation does not affect viral polymerase activity *(Figure 11)*, thus, the overall effect of this change on viral replication may be mediated by its decreased production of DGs, which may contribute to pathogenicity of CAL-PA recombinant virus *in vivo*. In addition to the ability of DGs production in infected cell, we observed that recombinant viruses were also able to produce DGs in *in vivo* infections *(Figure 30)*. Although not very strict, there is certain correlation between *in vivo* pathogenicity and DGs generation, since more pathogenic recombinant viruses produce low amount of DGs, such as CAL and CAL-PA recombinants. To ascertain the relationship between DGs generation and *in vivo* pathogenicity we compared the pathogenicity of recombinant viruses with clearly distinguishable DGs production. Introduction of the M1+M2 mutations on the CAL or CAL-PA backgrounds, which generate high accumulation of DGs, leads to decreased virulence attenuation in mice *(Figure 33b)*, as demonstrated by their

increased LD_{50}. Besides viral attenuation of these two viruses, CAL-M-PA virus pathogenicity was greater than that of CAL-M virus, as indicated by body weight loss after sub lethal infection *(Figure 335b)*. These data support the idea that reduced DGs accumulation is a pathogenic determinant for influenza virus in mice, and that mutation PA D529N derived from a fatal IAV case decreases DGs generation. DGs generation ability compared with replication of full-length segments inversely correlates with pathogenicity in mouse model. One exception is represented by CAL-HA recombinant virus which was completely attenuated in mice although it did not accumulate DGs throughout infection. As mentioned, mutation in HA viral protein of CAL-HA recombinant virus may be a host range factor and therefore it can be completely attenuated in mice. Thus, we cannot exclude that the CAL-HA virus would have a different phenotype when expressed in a permissive animal model.

Production of DGs has been correlated with morbidity and induction of antiviral response in patients infected with RSV *(Sun, Jain et al. 2015)* and mice infected with Sendai and influenza A virus *(Tapia, Kim et al. 2013)*. Viruses with diminished ability to produce DGs have a delay in activation of cellular antiviral response independently of the origin of the virus. However, influenza virus capacity of DGs generation and their possible correlation with pathogenicity in humans has never been evaluated. Several studies in our laboratory carried out an unbiased approach to detect pathogenicity markers for highly pathogenic influenza viruses. To evaluate the role of accumulation levels of DGs in the pathogenicity of influenza viruses circulating in humans, next generation sequencing (NGS) was used to analyze viruses isolated from respiratory samples from a cohort of A(H1N1)pdm09-infected patients admitted to intensive care unit (ICU) or deceased (Severe-Fatal cohort). For more precise characterization of the intrinsic pathogenicity of all these viruses, only those isolated from patients aged over 1 and under 65, and with no known comorbidities (only for ICU admitted patients) were included (Figure 1A). The cohort of ICU admitted patients is a faithful representation (80-100%) of the total confirmed fatal H1N1 influenza cases for these criteria in the 2013-2014 influenza season in Spain. These viruses were compared to those isolated from a cohort of IAV patients suffering mild symptoms (C, control).

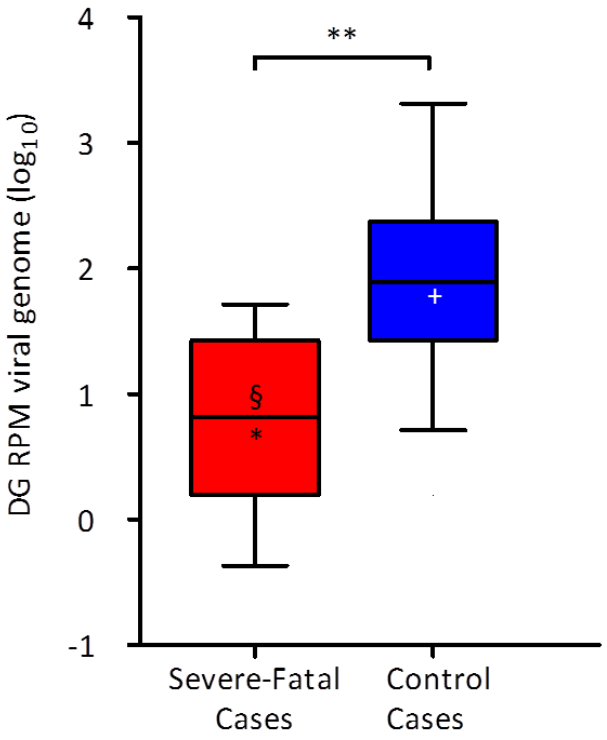


Figure 35. Defective genomes accumulation. DGs accumulated in *in vitro* infections with viruses originated from patients with severe-fatal outcome (red) and mild (blue) clinical outcome. DGs production by the F isolate (*); the M isolate (+); and the CAL recombinant virus (§).

Our study showed that the mean of DGs production of the severe-fatal (S-F) cohort *(Figure 35)*, is lower than the mean of clinical isolates obtained from control patients (C). Curiously, the control viruses isolated from patients suffering mild symptoms showed DGs production from every viral segment, whereas some of the viruses isolated from S-F patients lack DGs from polymerase segments (data not shown). The evaluation of specific amino acids changes from S-F viruses showed that changes affect different proteins. In addition, a small proportion of them did not show any amino acid change. The overall sequence analysis of these clinical strains did not give weight to any particular change in viral genome, but point out the lower nucleotide variability of strains isolated from the patients with a severe outcome, which accumulated fewer changes in the consensus sequence than strains isolated from control patients (database from NCBI) (manuscript under review). These results suggest that the low accumulation of DGs in S-F viruses might be mediated by different changes in distinct viral proteins, in the RNA structure, or by combinations of these elements. Additionally, these findings suggest that low DG abundance is a determinant of viral pathogenicity and has a role in the fatal outcome of influenza infection in humans independently of the amino acid or nucleotide changes responsible. Our results suggest quantitative determination of DGs as a prognostic marker for severe influenza disease.

1.3 Role of host factors on viral pathogenesis

Host factors may be important for pathogenicity in flu infections. Almost 300 host proteins have been involved in the early replication of influenza virus in mammalian cells, and many of them are involved in the highly delicate host-pathogen interaction network *(Konig, Stertz et al. 2010)*.

Study of human host factors and its role in influenza infection, has been focal point of extensive research in our lab, with major interests in the ones involved in transcription and replication of influenza virus. We have described that various cellular factors interact with polymerase; hCLE/C14orf166 which not only interacts with vRNPs both in nucleus and cytoplasm of infected cells, but is also incorporated in the viral particle *(Rodriguez-Frandsen, de Lucas et al. 2016)*; or NXP2/MORC3 that positively regulate influenza virus multiplication *(Ver, Marcos-Villar et al. 2015)*. Several members of the CHD family of chromatin remodelers, CHD1 and CHD6, associate with viral polymerase and act as positive and negative modulator of influenza virus replication, respectively *(Alfonso, Lutz et al. 2011, Marcos-Villar, Pazo et al. 2016)*. Also, host factors involved in protein translation such as the polyA binding protein 1 (PABP1) or the eIF4G have been characterized as interactors with influenza virus proteins. Recently, these host factors have been described as potential influenza antiviral targets *(Rodriguez, Perez-Morgado et al. 2016)*.

During 2009 pandemic, patients without any underlying medical conditions and similar health status suffered from different flu induced symptoms, ranging from mild illness to fatal outcome. Recent studies suggest that some genetic markers (SNP polymorphisms in inflammatory cytokines) could affect severity and final disease outcome in patients infected with influenza virus. The -238 A SNP (rs361525 A) allele of the TNF gene was described as risk factor for severe pneumonia *(Antonopoulou, Baziaka et al. 2012, Garcia-Ramirez, Ramirez-Venegas et al. 2015)* as well as IL6 (rs1818879 GA) heterozygous genotype *(Garcia-Ramirez, Ramirez-Venegas et al. 2015)*. Polymorphism in genes LTA (rs909253 AG), IL8 (rs4073 AA) contributes to disease severity in A(H1N1)pdm09 infected patients *(Morales-Garcia, Falfan-Valencia et al. 2012)* while IL1B (rs16944 AG and rs3136558 TC) SNPs decrease the risk of infection *(Garcia-Ramirez, Ramirez-Venegas et al. 2015)*.

One of these genetic markers is CCR5Δ32, a deleted form of *Ccr5* gene, which is associated with influenza severity in patients *(Falcon, Cuevas et al. 2015)*. C-C chemokine receptor (CCR5) is expressed on the surface of dendritic cells, macrophages, activated and memory T cells. HIV virus uses CCR5 receptor to target these immune cells and infect them. There are two alleles of *Ccr5* gene in human population, a wild type CCR5 and CCR5Δ32 allele which possess 32 base pair deletion that results in non-functional receptor. In HIV infection CCR5Δ32 heterozygous patients have slower progression of infection while homozygous patients show considerable resistance to some HIV subtypes *(Liu, Paxton et al. 1996)*. In influenza virus infection, patients with one or both deleted alleles of the CCR5 receptor (CCR5Δ32 homo- and heterozygous) were more susceptible to develop severe decease *(Rodriguez, Falcon et al. 2013, Falcon, Cuevas et al. 2015)*. Interestingly enough, patient infected with F viral strain (HA S127L, PA D529N, PB2 A221T) was also homozygous for CCR5Δ32 allele, and thus the increased pathogenicity of the infecting virus and the genetic background of the patient may have cooperated in the fatal outcome of infection *(Falcon, Cuevas et al. 2015)*.

All these findings suggest that, besides viral, host factors play an important role not only in early stages of infection but also at later steps, contributing to pathogenicity and virulence of influenza A virus and affecting the course of disease and its progression in patients.

1.4. Innate immune response contributes to viral pathogenicity in vivo

Appropriate host response provides early line of defence, which affects the progression of the infection and the development of illness. Innate immune response as first strategy against viral pathogen serves to prevent, control and

eliminate infection and operates by induction of two major types of response; the inflammation and the antiviral defence. Antiviral defence is a process by which infected cells counteract the viral replication and became more susceptible to lymphocytes recognition. Inflammation response consists of leukocyte influx and circulating plasma proteins in the infected tissue and its activation in order to eliminate viral antigens. Acute inflammation, besides elimination of virus infection, induces tissue damage. That is why an adequate host immune response, disease tolerance and inflammation, greatly affect disease advance and its final outcome *(Abbas, Lichtman et al. 2012)*.

1.4.1 Antiviral cellular response

Appropriate immune response (innate and adaptive) plays a key role in final outcome of infection. Sensing viral pathogen is a crucial step in the control of viral infection and development of illness. RIG-I is a major sensor of virus and therefore, essential in the control of viral infections. Upon the recognition of viral antigens, infected cells initiate several signalling cascades that induce intracellular antiviral and pro-inflammatory response. Antiviral response in infected cells induces transcription of type I interferons (IFN-α, β) which then activates transcription of interferon stimulated genes (ISGs) among them MxA and ISG56 (IFITM 1) proteins *(Goodbourn, Didcock et al. 2000)*. These antiviral proteins suppress replication and transcription of viral genome in infected cells *(Kochs and Haller 1999)* and block viral entry *(Bailey, Zhong et al. 2014)*, respectively. Our results suggest that viruses with high DGs accumulation capability induce stronger antiviral response in early infection *in vitro* *(Figure 26, 27a, b, and c)* in accordance with previous study *(Tapia, Kim et al. 2013).* We observed that highly pathogenic CAL-PA recombinant virus induces lower transcription of MxA and ISG56 antiviral genes *(Figure 27a, b)* at the very beginning of infection. These differences disappear after the end of the first viral cycle. Differences in activation of antiviral response at early times of infection may be explained by the dissimilar capability for DGs generation of the different recombinant viruses. Pattern recognition receptor, RIG-I possesses a high binding affinity for short, ssRNA molecules *(Kato, Takeuchi et al. 2008)* such are DGs molecules up to 1000 nt long. This recognition activates signal cascades that terminate in IFN-α/β dependent activation of antiviral gene transcription. Induction of IFN-β transcription, in infection with more pathogenic recombinant viruses (CAL, CAL-PB2/PA and CAL-PA), does not correlate with ISG56 and MxA expression at early stage of infection *(Figure 27)*. This may indicate that IFN-β is not the prominent inducer of antiviral gene transcription in viral infections with these recombinant viruses. Since IFN-λ have been described as one of the crucial activators of antiviral response in influenza infection in both cell culture *(Wang, Oberley-Deegan et al. 2009)* and mouse model *(Kim, Kim et al. 2016)*, IFN-λ may be the actual activators of antiviral gene transcription. However, further experiments will be required to evaluate this proposed data.

As previously mentioned, not just the quantity but also the quality of DGs may affect overall antiviral response in infected cells. Our reference, CAL recombinant virus generates the same amount of DGs as the highly pathogenic CAL-PA virus, yet induces higher antiviral response than CAL-PA at early stage of infection. Having in mind that CAL recombinant virus produces DGs almost exclusively originated from NS segment, which would correspond to defective NS1 mRNA, it would imply that CAL recombinant virus with certain amount of non-functional NS1 protein would not be able to efficiently impair antiviral response *(Gack, Albrecht et al. 2009)* and therefore, will induce more ISG56 and MxA than CAL-PA virus.

Considering these data in more complex in vivo system, a hampered sensing of early viral infection by the involved cellular sensors will delay the activation of appropriate antiviral response and will promote immune tolerance to infection, allowing higher viral replication rates in early infections. On the flip side, uncontrolled viral growth would lead to enormous viral burden that would induce excessive inflammation process, which would clearly contribute to pathogenicity of the virus.

1.4.2 Cellular innate immune response (cell influx and inflammation)

Upon influenza infection, both immune response of the host and the nature of the viral strain are very important for the final outcome. Inadequate host innate immune response, severe cell influx, inflammation (pro-inflammatory cytokine storm) or immune tolerance to pathogen that leads to massive viral growth, could lead to fatal outcome. In viral infections, neutrophils and alveolar macrophages play a key role in clearance and control of viral growth in infected lungs, thus their substantial influx in infected tissue contributes to overall viral pathogenicity. Depletion of alveolar macrophages leads to an uncontrolled viral proliferation and fatal outcome in infected mice *(Tumpey, Garcia-Sastre et al. 2005)* while high influx of neutrophils in lungs and excessive inflammation has been associated with severe illness and high mortality rate in influenza infection *(Brandes, Klauschen et al. 2013)*. Therefore, excessive innate immune response and inflammation is observed in infection with highly pathogenic viral strains.

We found that recombinant viruses with PA D529N change (CAL-PA and CAL-PB2/PA) induce high infiltration of cells of innate immune response in lungs of infected animals *(Figure 22-25)* as well as severe inflammation *(Table 8)* caused by overexpression of chemokines, pro-inflammatory cytokines on the 2nd day of infection. Biomarkers of acute inflammation processes in lungs of patients with chronic obstructive pulmonary disease (COPD) *(Calero, Arellano et al. 2014)* were highly expressed in lung of CAL-PA infected mouse suggesting severe pulmonary damage in infected animal. Alveolar macrophages showed significant depletion on the 2nd day of infection *(Figure 23)* in CAL-PA infected lungs which was a general feature for all previously described pathogenic recombinant viruses *(Table 6)*. This depletion of alveolar macrophages perfectly correlates with increased viral titers in lung tissue of infected animals *(Figure 16)*, emphasizing again the crucial role of these cells in viral clearance and control of viral growth. Our findings show that CAL-PA virus is a potent inductor of innate immune response (cellular influx) and extensive inflammation process in infected lungs, which greatly contributes to its pathogenicity *in vivo*. Since it has been described that PA subunit of viral polymerase is one of the markers of pathogenicity of influenza A viruses as well as an inducer of strong innate immune response and inflammation of infected lungs *(Hu, Hu et al. 2013)*, PA D529N change in polymerase complex may enhance severe host innate immune response (cell influx and inflammation). Both viruses that contain this mutation (CAL-PA and CAL-PB2/PA) have similar influx of innate immune cell response although they do not have the similar viral load *(Figure 16)* on the same day of infection (2nddpi). Therefore, immune response detected in animals infected with these viruses does not correlate with observed lung viral titer in these animals. What is more, CAL-PB2/PA has similar viral growth in infected lungs as CAL recombinant virus *(Figure 16)*, while animals infected with these recombinants have a completely different cell influx innate immune response *(Figure 22-25)*. Together, these data suggest that D529N change in PA viral polymerase subunit is responsible for increased induction of innate immune response (cellular influx) and extensive inflammation process independently of viral burden in infected lungs.

Since viruses with D529N mutation in PA subunit of viral polymerase induce strong innate immune response and inflammation in infected animals independently of its high growth capability in lungs, high viral growth *in vivo* might not be solely dependent of the mutation found in PA subunit but trough depletion of alveolar macrophages in infected lungs *(Figure 23)* as well. Alveolar macrophages, besides pneumocytes and dendritic cells *(Hartmann, Thakar et al. 2015)*, are the infectious target of highly pathogenic influenza strains (Kasloff and Weingartl 2016), and its prominent infection can compromise viral and tissue damaged clearance, as well as adequate early innate immune response *(Chang, Kuchipudi et al. 2015, Halstead and Chroneos 2015)*.

Therefore, high viral titers observed in recombinants with PA D529N change may depend on various factors that contribute to high viral growth in infected lungs. Initial delay in antiviral cellular response in very early stage of infection may allow unhampered viral growth in infected cells. As a consequence of low antiviral cell state and undisturbed viral growth in early infection, neighbouring pneumocytes as well as alveolar macrophages and other cells involved in fast viral infection response would not only suffer a delay in activation of innate cellular immune response but also become an easy viral targets in first hours of infection. However, after the first several hours of infection, in later stages of infection extreme viral burden would provoke potent and vigorous immune response causing severe cell influx, acute inflammation and serious and massive damage in infected tissue. This infection pattern precisely corresponds to our observations obtained in the murine infection model with PA D529N mutant viruses.

1.5 Viral spread promotes viral pathogenicity in vivo

Viral spread represents an important aspect of overall pathogenicity of any influenza viral strain. In critically ill patients several clinical complications have been associated with cardiac abnormalities *(Jeyanathan, Overgaard et al. 2013)*, renal *(Watanabe 2013)* and neurological *(Frobert, Sarret et al. 2011)* difficulties. Influenza infection in some fatal cases induces different heart complications, inflammation in hearts, presence of the viral particles in this organ *(Komai, Nakazawa et al. 2011, Jeyanathan, Overgaard et al. 2013)* and severe hypoxemia in patients in critical medical condition *(Quddus, Afari et al. 2015)*. Very common complication of A(H1N1)pdm09 infections were transient and reversible cardiac dysfunctions *(Martin, Hollingsworth et al. 2010),* which emphasizes the capability of viral spread of influenza A virus as a pathogenicity marker. Furthermore, infection of mice with pathogenic A/Puerto Rico/8/34 strain leads to heart infection that induces inflammation in this organ as well as presence of viral particles up to 9 days upon the infection *(Pan, Sun et al. 2014)*.

Using recombinant viruses containing specific mutations in the viral polymerase, we showed that infection with CAL-PA recombinant results in high viral titers in infected mice lungs during the first four days of infection *(Figure 16)*. Interestingly enough, apart from usual influenza infection symptoms such as weight loss, shivers, lethargy, etc. we observed a phenomenon of sudden death at very early stage of infection (3dpi) in CAL-PA infected mice with high viral doses (10^6 and $3x10^5$ pfu). Sudden death usually occurs from cardiac causes, and here we have detected presence of viral particles due to genuine replication in heart tissue. Incidence of hearts positive for viral particles detection of animals infected with CAL-PA virus sums up to 80% by the 4^{th} day of infection *(Figure 19)*. This result indicates that CAL-PA recombinant virus has stronger capability for heart infection than other recombinant viruses. Sample contamination was discarded as origin of viral presence in the heart since the heart titers do not correlate or depend

on the replication in lungs *(Figure 21)*; for instance some mice having high viral titers in lung do not present infectious particles in the hearts. Therefore, persistent and prolonged heart infections as well as ongoing viral replication in heart tissue in early infection contribute to pathogenicity of CAL-PA virus, especially having in mind that viral doses used for this infection assay are sub lethal.

To evaluate whether viral presence in the heart could induce a physiological cardiac damage, we have conducted a preliminary study in collaboration with cardiologists from Centre for Arrhythmia Research at university of Michigan, College of Medicine Cardiology at University of South Florida, and CNIC, Madrid. Electrocardiogram (ECG) was monitored on mock-infected animals and mice infected with high viral doses (10^6 pfu) of CAL-PA or CAL-PB2 recombinant virus for 4 days after infection and compared with their own uninfected cardiac status. The ECGs analysis shows that animals infected with both viruses suffered from bradycardia. Additionally, only those animals infected with CAL-PA recombinant virus *(Figure 36)* showed cardiac conduction defects, which correlate with viral particles presence in hearts all throughout the infection. Further studies would give us more insights into influenza virus effect on heart conduction system as well as its effect on progression of disease and final outcome.

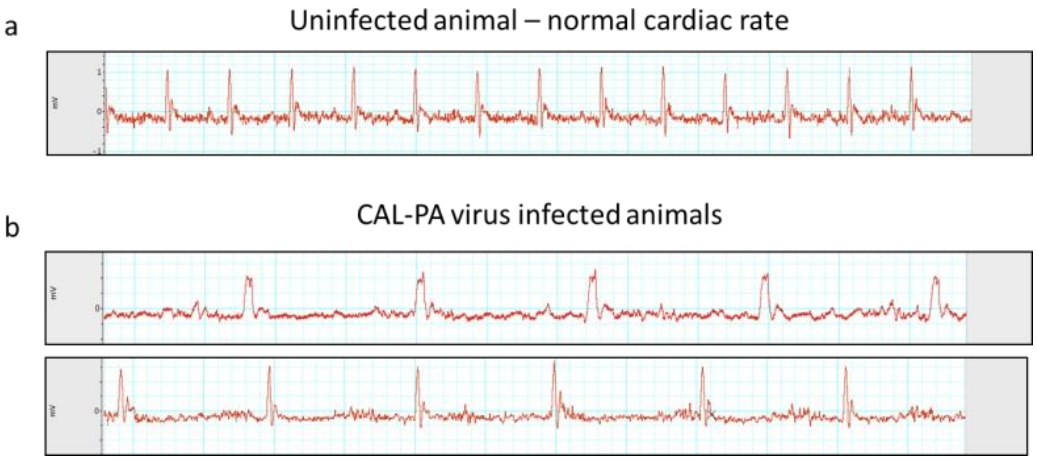


Figure 36. Representative electrocardiogram of control or flu infected animals. a) Cardiac rate of an uninfected animal on the 2nd dpi. (b) Cardiac rate of two representative animals infected with high viral dose (10^6 pfu) of CAL-PA recombinant virus on the 2nd dpi, in which enhanced systolic bradycardia and conduction disorders have been observed.

Another virus with positive sense ssRNA genome, the Coxsackievirus B (CVB), affects heart tissue and causes myocarditis, which might be associated with heart failure and left ventricular dysfunction. Infection and replication of this virus induces cardiomyocytes injury and activates maladaptive inflammatory responses *(Garmaroudi, Marchant et al. 2015)*. 5´ deletions of RNA genome of CVB found in serial passages *in vitro* as well as *in vivo* (infected mice hearts) affects viral replication in infected cells by accumulation (–) RNA genome *(Kim, Chapman et al. 2008)*. Persistence of defective genome was detected 5 months upon viral infection in mice, without the necrotic effects typically observed during infection with virus with intact genome *(Kim, Tracy et al. 2005)*. Deletions in viral genome allow CVB to persist in the resident tissue expressing non-cytolytic phenotype and prolonging lower inflammatory response than viruses with intact RNA genome. A similar mechanism may operate in influenza virus heart infection. CVB induces fulminant myocarditis in patients, which causes several heart dysfunctions by induction of tissue damage; influenza virus

infection may have the same *modus operandi*. CAL-PA recombinant virus infects and replicates in heart tissue of infected mice, and induces excessive innate immune response and severe inflammation in infected lungs. Moreover, this recombinant virus possesses a mutation in the PA subunit of viral polymerase that affects production of DGs and overall viral replication fidelity, allowing CAL-PA virus to replicate more efficiently (production of intact full length segments) than other recombinant viruses. High viral replication and persistence of this virus in heart tissue *(Figure 20 and 21)* may induce severe myocarditis in infected animals and cause phenomenon of sudden death that we observed. Furthermore, our findings suggest that influenza A virus can infect Purkinje fibers in heart besides cardiomyocytes. Preliminary analysis of histological samples of heart tissue of mice infected with clinical isolate from a deceased patient, F virus, seem to indicate that along with cardiomyocyte, Purkinje fibers were also infected with influenza virus. Normal heart sinus rhythm is maintained by natural heart pacemakers sinoatrial (SA node) and atrioventricular (AV node) nodes and Purkinje fibers. Dysfunction of pacemaker cells in the nodes or Purkinje fibers causes irregular heart rhythm and viral infection of these cells may provoke abnormal heart rhythm (arrhythmia). Further investigation regarding influenza virus heart infection and its mechanisms are necessary in order to complete understanding of this complex process.

As it was mention before, pathogenicity of any viral strain is a multifactorial process that depends on various viral and host factors as well as delicate interplay between them. In our study we showed that a single mutation in the viral polymerase complex can affect several aspects of *in vivo* infection. D529N change in PA polymerase subunit allowed high viral growth in the lung of infected mice, induced stronger innate immune response and enhanced high viral replication and persistence in hearts of infected animals. Mutation PA D529N was detected in a clinical strain isolated from a deceased patient and may be a key factor to pathogenicity of H1N1 influenza A virus in humans.

1. A221T aa change in PB2 subunit of the viral polymerase is an attenuating factor in the context of an Influenza A(H1N1)pdm09 virus in *in vivo* model.

2. S127L aa change in HA surface glycoprotein does not contribute to virulence of the F virus isolated from a fatal case and it may be considered a host restrictive range factor for mice.

3. Introduction of PA D529N aa change in A/California/04/09 strain backbone virus decreases almost 100-fold LD_{50} *in viv*o.

4. D529N aa change in PA subunit of the viral polymerase is the major contributor to the high pathogenicity of the F virus isolated from a fatal case.

5. Recombinant virus containing PA D529N aa change confers virulence and pathogenesis through:

    - low accumulation of DGs during *in cell culture* infections

    - low induction of antiviral response in very early stage of *cell culture* infection

    - high viral replication in the lungs of infected animals

    - excessive innate immune cell influx (neutrophils and presumably conventional dendritic cells) and acute inflammation observed in lungs of animals infected with sub lethal viral doses

    - higher replication and prolonged presence of infectious viral particles in hearts of infected animals which seems to be associated with cardiac disorders observed in infected animals

6. Decreased accumulation of defective genomes found in A/California/04/09 recombinant viruses containing PA 529N (CAL-PA, CAL-PB2/PA, CAL-M/PA) confers increased pathogenicity in the mice model, which is in accordance with pathogenic viral phenotype of natural viral isolates found in hospitalized patients with severe-fatal outcome.

1. El cambio de aa A221T en la subunidad PB2 de la polimerasa viral es un factor de atenuación en el contexto de un virus de la gripe A(H1N1)pdm09 en un modelo *in vivo*.

2. El cambio de aa S127L en la glicoproteína de superficie HA, no contribuye a la virulencia del virus F aislado de un caso fatal y puede considerarse un factor de rango de huésped en ratón.

3. La introducción del cambio D529N en la subunidad PA en la cepa A/California/04/09 disminuye casi 100 veces la LD_{50} *in vivo*.

4. El cambio de aa D529N en la subunidad PA de la polimerasa viral supone la mayor contribución a la alta patogenicidad del virus F aislado de un caso fatal.

5. El cambio de aa PA D529N en un virus recombinante confiere virulencia y patogenicidad a través de:

    - baja acumulación de DGs durante la infección en células en cultivo

    - baja inducción de respuesta antiviral en estadios muy tempranos de la infección en células en cultivo

    - alta replicación viral en los pulmones de los ratones infectados

    - excesivo reclutamiento (neutrófilos y presumiblemente células dendríticas convencionales) e inflamación aguda observada en los pulmones de los ratones infectados con dosis subletales

    - alta replicación y presencia prolongada de partículas virales en los corazones de los animales infectados, lo que parece estar asociado con desordenes cardíacos observados en animales infectados

6. La disminución de genomas defectivos encontrada en virus recombinantes A/California/04/09 conteniendo PA D529N (CAL-PA, CAL-PB2/PA, CAL-M/PA) confiere patogenicidad incrementada en el modelo de ratón, lo que está de acuerdo con el fenotipo de patogenicidad encontrado en aislados virales de pacientes hospitalizados con un desenlace severo-fatal.

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